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(54) Title: PEPTIDES THAT MODULATE THE INTERACTION OF B CLASS EPHRINS AND PDZ DOMAINS

(57) Abstract

The invention relates to complexes comprising a B class ephrin and a PDZ domain containing protein; peptides that interfere with the interaction of a B class ephrin with a PDZ domain binding site, and a PDZ domain containing protein; and, uses of the peptides and complexes. Methods for modulating the interaction of a B class ephrin and a PDZ domain containing protein, and methods for evaluating compounds for their ability to modulate the interaction are also described.

**TITLE: Peptides that Modulate the Interaction of B class Ephrins and PDZ Domains**

## FIELD OF THE INVENTION

The invention relates to complexes comprising a B class ephrin with a PDZ domain binding site, and a PDZ domain containing protein; peptides that interfere with the interaction of a B class ephrin with a PDZ domain binding site, and a PDZ domain containing protein; and, uses of the peptides and complexes.

## BACKGROUND OF THE INVENTION

Among the large number of receptor tyrosine kinases (RTK) identified in metazoan organisms, the members of the Eph family are unusual in several respects. Although only one Eph RTK is known to be encoded by the *Caenorhabditis elegans* genome (the *vab-1* gene product (2)), vertebrates typically possess up to 14 genes for Eph receptors, suggesting that these tyrosine kinases may be important in controlling complex cellular interactions (3,4). Consistent with this possibility, *C. elegans* VAB-1 regulates morphogenetic cell movements during ventral closure in the embryo (2), while vertebrate Eph receptors have been implicated in controlling axon guidance and fasciculation, in specifying topographic map formation within the central nervous system, in organizing the movements of neural crest cells during development, in directing fusion of epithelial sheets in closure of the palate, and in angiogenesis (5-15).

Early work on the expression patterns of EphB2 (formerly Nuk) suggested that this receptor is clustered at sites of cell-cell junctions in the developing mouse mid-brain, and raised the possibility that Eph receptors might mediate signals initiated by direct cell-cell interactions (5). Several lines of evidence support the notion that Eph receptors are normally activated by ligands that are physically associated with the surface of an adjacent cell. All known ligands for the Eph receptors (termed ephrins) are related in sequence, but can be divided into two groups based on their C-terminal motifs. The ephrin A class of ligands become modified by a C-terminal glycosylphosphatidyl inositol (GPI) moiety, through which the ligand is anchored to the surface of the ligand-expressing cell (7,9,16). In contrast, B-type ephrins possess a transmembrane element, and a highly conserved cytoplasmic tail comprised of 82-88 C-terminal residues (17-22). The Eph receptors can, in turn, be divided into A and B subgroups based on their sequence similarity and their propensity to bind soluble forms of either A or B type ephrins, respectively (4,23,24). However, although soluble ephrins bind tightly to the relevant receptors, consistent activation of Eph tyrosine kinase activity requires either that the ligands be artificially clustered into oligomers, or that receptor-expressing cells be co-cultured with cells expressing membrane-associated ephrins (18). These data suggest that the ability of ephrins to aggregate and thereby activate Eph receptors depends on their attachment to the cell surface, consistent with the view that Eph receptor signaling involves cell-cell interactions. During embryonic development in the mouse, Eph receptors and their ligands are expressed in dynamic but complementary patterns, indicating that Eph receptors are likely activated at boundaries where Eph and ephrin-expressing cells are directly juxtaposed to one another (23, 25).

Genetic analysis of Eph receptor function in *C. elegans* and the mouse has indicated that Eph receptors have both kinase-dependent and kinase-independent modes of signaling, and raised the possibility that B-type Eph receptors and ephrins might mediate bi-directional cell-to-cell signaling (2,6). Of interest, the binding of Eph receptors to transmembrane ephrin B1 or ephrin B2, as well as treatment of ephrin B-expressing cells with platelet-derived growth factor (PDGF), leads to the phosphorylation of the ephrins on tyrosine residues within their highly conserved cytoplasmic tails (26,27). Furthermore, expression of the cytoplasmic tail of a *Xenopus* ephrin B molecule leads to a striking loss of cell adhesion in *Xenopus* embryos, an effect that is suppressed by treatment with fibroblast growth factor (28).

**SUMMARY OF THE INVENTION**

B class ephrins function as ligands for B class Eph receptor tyrosine kinases and possess an intrinsic signaling function. The sequence at the carboxy-terminus of B-type ephrins contains a PDZ binding site, providing a mechanism through which transmembrane ephrins interact with cytoplasmic proteins. A day 10.5 mouse embryonic expression library was screened with a biotinylated peptide corresponding to the C-terminus of ephrin B3. Three of the positive cDNAs encoded polypeptides with multiple PDZ domains, representing fragments of the molecule GRIP, the protein syntenin and PHIP, a novel PDZ domain-containing protein related to *Caenorhabditis elegans* PAR-3. In addition, the binding specificities of PDZ domains previously predicted by an oriented library approach (1) identified the tyrosine phosphatase FAP-1 as a potential binding partner for B ephrins. *In vitro* studies demonstrated that the fifth PDZ domain of FAP-1 and full-length syntenin bound ephrin B1 via the C-terminal motif. Lastly, syntenin and ephrin B1 could be co-immunoprecipitated from transfected Cos-1 cells, indicating that PDZ domain binding of B ephrins occurs in cells. These results indicate that the C-terminal motif of B ephrins provides a binding site for specific PDZ domain-containing proteins, which potentially localize the transmembrane ligands for interactions with Eph receptors or participate in signaling within ephrin B-expressing cells.

Broadly stated the present invention relates to a complex comprising a B class ephrin and a PDZ domain containing protein. The invention is also directed to a peptide derived from the PDZ binding domain of a B class ephrin. The invention also contemplates antibodies specific for the complexes and peptides of the invention.

The present invention also provides a method of modulating the interaction of a B class ephrin and a PDZ domain containing protein comprising administering an effective amount of one or more of the following: (a) a complex comprising a B class ephrin and a PDZ domain containing protein; (b) a peptide derived from the PDZ binding domain of a B class ephrin; or, (c) enhancers or inhibitors of the interaction of a B class ephrin and a PDZ domain containing protein.

The invention still further provides a method for identifying a substance that binds to a complex comprising a B class ephrin B and a PDZ domain containing protein comprising: (a) reacting the complex with at least one substance which potentially can bind with the complex, under conditions which permit binding of the substance and complex; and (b) detecting binding, wherein detection of binding indicates

the substance binds to the complex. Binding can be detected by assaying for substance-complex conjugates, or for activation of the B class ephrin B or PDZ domain containing protein. The invention also contemplates methods for identifying substances that bind to other intracellular proteins that interact with the complexes of the invention.

Still further the invention provides a method for evaluating a compound for its ability to modulate the interaction of a B class ephrin and a PDZ domain containing protein. For example, a substance that inhibits or enhances the interaction of the molecules in a complex of the invention, or a substance which binds to the molecules in a complex of the invention may be evaluated. In an embodiment, the method comprises providing a complex of the invention, with a substance which binds to the complex, and a test compound under conditions which permit the formation of conjugates between the substance and complex, and removing and/or detecting conjugates. In another embodiment, the method comprises providing a B class ephrin and a PDZ domain containing protein, and a test compound, under conditions which permit binding of the B class ephrin and PDZ domain containing protein; and (b) detecting binding, wherein the detection of increased or decreased binding relative to binding in the absence of the test compound indicates that the test compound modulates the interaction of a B class ephrin and a PDZ domain containing protein.

The present invention also contemplates a peptide of the formula I which interferes with the interaction of a B class ephrin and a PDZ domain containing protein

$$X\text{-}X^1\text{-}X^2\text{-}K\text{-}V \qquad \text{I}$$

wherein X represents 0 to 70, preferably 0 to 50, more preferably 2 to 20 amino acids, and $X^1$ and $X^2$ each represent tyrosine or phosphotyrosine. The invention also relates to analogs of the peptides of the invention.

Further, the invention relates to a method of modulating the interaction of a B class ephrin and a PDZ domain containing protein comprising changing the terminal amino acid Val in a B class ephrin.

The complexes, peptides and antibodies of the invention, and substances and compounds identified using the methods of the invention may be used to modulate the interaction of a B class ephrin and a PDZ domain containing protein, and they may be used to modulate cellular processes of cells associated with B class ephrins and/or PDZ domain containing proteins (such as proliferation, growth, and/or differentiation, in particular axonogenesis, nerve cell interactions and regeneration) in which the compounds or substances are introduced.

Accordingly, the complexes, antibodies, peptides, substances and compounds may be formulated into compositions for administration to individuals suffering from disorders associated with a B class ephrin such as disorders of the central nervous system (e.g. neurodegenerative diseases and cases of nerve injury). Therefore, the present invention also relates to a composition comprising one or more of a complex, peptide, or antibody of the invention, or a substance or compound identified using the methods

of the invention, and a pharmaceutically acceptable carrier, excipient or diluent. A method for modulating proliferation, growth, and/or differentiation of cells associated with B class ephrins and/or PDZ domain containing proteins is also provided comprising introducing into the cells a complex, peptide or antibody of the invention, a compound or substance identified using the methods of the invention or a composition containing same. Methods for treating proliferative and/or differentiative disorders associated with B class ephrins and/or PDZ domain containing proteins using the compositions of the invention are also provided.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

Fig. 1. shows the amino acid sequence of the cytoplasmic domains of the human B ephrins (SEQ. ID. NOS. 15, 16, and 17);

Fig. 2A. shows a preferred binding sequence of FAP-1 PDZ5 (SEQ ID. NO.18, 19, and 20) below a schematic representation of the entire FAP-1 protein tyrosine phosphatase;

Fig. 2B are diagrammatic representations of the PDZ domain-containing proteins identified through an expression screen with a biotinylated peptide probe of ephrin B3 C-terminal sequence;

Fig. 2C shows amino acid sequence alignment of FAP-1 PDZ5 and of the PDZ domains isolated in the expression screen (SEQ. ID. NO. 21 to 27);

Fig. 2D shows the amino acid sequence alignment of PHIP (SEQ ID. NO. 1 ) and PAR-3 (SEQ. ID. NO. 34);

Fig. 3A is a blot showing the binding of FAP-1 PDZ5 GST fusion proteins to ephrin B1;

Fig. 3B is a blot showing the binding of FAP-1 PDZ5 fusion proteins to ephrin B1;

Fig. 3C is a blot showing the binding of syntenin GST proteins to ephrin B1;

Fig. 3D is a blot showing the binding of syntenin GST proteins to ephrin B1;

Fig. 4A is a blot showing blocking of FAP-1 PDZ5 binding to ephrin B1 by addition of peptides corresponding to the C-terminal sequence of B ephrins;

Fig. 4B is a blot showing blocking of syntenin binding to ephrin B1 by addition of peptides corresponding to the C-terminal sequence of B ephrins;

Fig. 5A is a graph showing fluorescence polarization analysis of GST-FAP-1 PDZ3, and GST-FAP-1 PDZ5 binding to fluorescein-labeled peptides corresponding to the C-terminus of ephrin B1;

Fig. 5B is a graph showing fluorescence polarization analysis of GST-syntenin binding to fluorescein-labeled peptides corresponding to the C-terminus of ephrin B1;

Fig. 6. is a blot showing co-immunoprecipitation of syntenin-FLAG with ephrin B1;

Fig. 7 is a graph showing a fluorescence polarization analysis of GST-PHIP PDZ3 binding to fluorescein-labelled peptides corresponding to the C-terminus of ephrin B1; and

Fig. 8 is an immunoblot showing that PHIP PDZ3 binds specifically to V-Src phosphorylated ephrin B1 in GST-mixes.

## DETAILED DESCRIPTION OF THE INVENTION

**Definitions**

Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Current Protocols in Molecular Biology (Ansubel) for definitions and terms of the art.

Abbreviations for amino acid residues are the standard 3-letter and/or 1-letter codes used in the art to refer to one of the 20 common L-amino acids. Likewise abbreviations for nucleic acids are the standard codes used in the art.

"Antibody" refers to intact monoclonal or polyclonal molecules, and immunologically active fragments (e.g. a Fab or $(Fab)_2$ fragment), an antibody heavy chain, and antibody light chain, a genetically engineered single chain $F_v$ molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art. Antibodies that bind a complex, or peptide of the invention can be prepared using intact peptides or fragments containing an immunizing antigen of interest. The polypeptide or oligopeptide used to immunize an animal may be obtained from the translation of RNA or synthesized chemically and can be conjugated to a carrier protein, if desired. Suitable carriers that may be chemically coupled to peptides include bovine serum albumin and thyroglobulin, keyhole limpet hemocyanin. The coupled peptide may then be used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

"B class ephrin" refers to a family of proteins that bind Eph receptors and possess a transmembrane element, and a highly conserved cytoplasmic tail comprised of 82-88 C-terminal residues (17-22). Examples of B class ephrins include ephrin B1 (also known as LERK-2, Elk-L, EFL-3, Cek-L, and STRA1), ephrin B2 (also known as Htk-L, ELF-2, LERK-5, and NLERK-1), and ephrin B3 (also known as NLERK-2, Elk-L3, EFL-6, ELF-3, and LERK-8). The family also includes proteins with substantial sequence identity (i.e. homologs) and portions of the proteins (e.g. see SEQ. ID. NO. 15, 16, or 17). The B class ephrins used in the complexes and methods of the invention contain a binding domain that binds a PDZ domain containing protein. The binding domain contains the consensus sequence YYKV.

The term "isolated", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

The term " modulate", as used herein, refers to a change or an alteration in the biological activity of a protein. Modulation may be an increase or a decrease in protein activity, a change in binding

characteristics, or any other change in the biological, functional, or immunological properties of a protein.

The term "agonist" as used herein, refers to a molecule which when bound to a complex of the invention or a molecule in the complex, increases the amount of, or prolongs the duration of, the activity of a B class ephrin or PDZ domain containing protein, or increases complex formation. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that bind to a complex or molecule of the complex. Agonists also include a peptide or peptide fragment derived from the PDZ binding domain of a B class ephrin but will not include the full length sequence of the wild-type molecule. Peptide mimetics, synthetic molecules with physical structures designed to mimic structural features of particular peptides, may serve as agonists. The stimulation may be direct, or indirect, or by a competitive or non-competitive mechanism.

The term "antagonist", as used herein, refers to a molecule which, when bound to a complex of the invention or a molecule in the complex, decreases the amount of or duration of the activity of a B class ephrin or PDZ domain containing protein, or decreases complex formation. Antagonists may include proteins, nucleic acids, carbohydrates, or any other molecules that bind to a B class ephrin or PDZ domain containing protein. Antagonists also include a peptide or peptide fragment derived from the PDZ binding domain of a B class ephrin but will not include the full length sequence of the wild-type molecule. Peptide mimetics, synthetic molecules with physical structures designed to mimic structural features of particular peptides, may serve as antagonists. The inhibition may be direct, or indirect, or by a competitive or non-competitive mechanism.

"PDZ domain containing protein" refers to proteins or peptides, or parts thereof which comprise or consist of a characteristic structural motif known as the PDZ domain. (See the Structural Classification of Proteins (SCOP) database for the characteristics of the domain.) Examples of the proteins include GRIP, syntenin, and FAP-1, and homologs or portions thereof. Other proteins containing PDZ domains may be selected using public databases such as GENPEPT and ENTREZ. The present inventors isolated a novel PDZ domain containing protein designated "PHIP" as more particularly described herein. Examples of PDZ domain containing proteins include GRIP, GRIP PDZ6 and PDZ 7 of SEQ.ID.NO. 22 and 23, FAP-1 PDZ5 of SEQ. ID. NO. 21, amino acids residues 1 to 299 of syntenin, syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27, PHIP PDZ2 of SEQ. ID. NO. 24 , and PHIP PDZ3 of SEQ. ID. NO. 25.

A "binding domain" is that portion of the molecule in a complex of the invention which interacts directly or indirectly with another molecule in a complex of the invention. The binding domain may be a sequential portion of the molecule i.e. a contiguous sequence of amino acids, or it may be conformational i.e. a combination of non-contiguous sequences of amino acids which when the molecule is in its native state forms a structure that interacts with another molecule in a complex of the invention.

By being "derived from" a binding domain is meant any molecular entity which is identical or substantially equivalent to the native binding domain of a molecule in a complex of the invention. A peptide derived from a specific binding domain may encompass the amino acid sequence of a naturally occurring binding site, any portion of that binding site, or other molecular entity that functions to bind to

an associated molecule. A peptide derived from such a binding domain will interact directly or indirectly with an associated molecule in such a way as to mimic the native binding domain. Such peptides may include competitive inhibitors, peptide mimetics, and the like.

The term "interacting" refers to a stable association between two molecules due to, for example, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions. Certain interacting molecules interact only after one or more of them has been stimulated. For example, a PDZ domain containing protein may only bind to a substrate if the substrate is phosphorylated (eg. phosphorylated).

"Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review ). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a peptide, or agonist or antagonist of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide, or agonist or antagonist of the invention.

The following terms are used to describe the sequence relationships between two or more nucleic acid molecules or proteins: "reference sequence", and "substantial sequence identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, a segment of a full-length cDNA or gene sequence given in a sequence listing or may comprise a complete cDNA or gene sequence. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (USA) 85:2444, or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis; ClustalW program (55); and the Genestream Align Program). As applied to polypeptides, the term " substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap share at least 90 percent sequence identity, preferably at least 95 percent sequence identity, more preferably at least 99 percent sequence identity or more. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

**Complexes of the Invention**

The complexes of the invention comprise a B class ephrin protein and a PDZ domain containing protein. It will be appreciated that the complexes may comprise only the binding domains of the interacting molecules and such other flanking sequences as are necessary to maintain the activity of the complexes.

In an embodiment of the invention, the PDZ domain containing protein in the complex is GRIP, GRIP PDZ6 and PDZ 7 of SEQ.ID.NO. 22 and 23, FAP-1 PDZ of SEQ. ID. NO. 21, amino acids residues 1 to 299 of syntenin, syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27, PHIP PDZ2 of SEQ. ID. NO. 24, and PHIP PDZ3 of SEQ. ID. NO. 25. Examples of complexes of the invention include ephrin B3/GRIP; ephrin B3/GRIP PDZ6 and PDZ 7 of SEQ.ID.NO. 22 and 23; ephrin B1/FAP-1 PDZ of SEQ. ID. NO. 21; ephrin B1 or B3/amino acids residues 1 to 299 of syntenin; ephrin B1 or B3/ syntenin PDZ1 and PDZ2 of SEQ. ID. NO.26 and 27; ephrin B1 or B3/PHIP PDZ2 of SEQ. ID. NO. 24, and ephrin B1 or B3/PHIP PDZ3 of SEQ. ID. NO. 25. The complexes may comprise a portion of the B class ephrin, or a peptide of the invention. For example, the complex may comprise YYKV (SEQ ID. NO. 5), GPPQSPPNIpYYKV (SEQ ID. NO. 6), NIpYpYKV (SEQ ID. NO. 7), NIpYYKV (SEQ ID. NO. 8), NIYpYKV (SEQ ID. NO. 9), NIYYKV (SEQ ID. NO. 10), GNIYYKV (SEQ ID. NO. 28), GNIpYpYKV (SEQ ID. NO. 29), GNIpYYKV (SEQ ID. NO. 30), and GNIYpYKV (SEQ ID. NO. 31). Examples of such complexes include FAP-1 PDZ/NIYYKV, syntenin/NIYYKV, syntenin PDZ1 and PDZ2/NIYYKV, PHIP PDZ3/GNIYYKV, and PHIP PDZ2/GNIYYKV.

As illustrated herein the B class ephrin or portion thereof, or peptide of the invention, in a complex of the invention may be phosphorylated. Therefore, a complex of the invention comprising a PDZ domain containing protein as one component may comprise a phosphorylated B class ephrin or a portion thereof, or a phosphorylated peptide of the invention as another component. For example, the complex may comprise FAP-1 PDZ/NIpYYKV, FAP-1 PDZ/NIpYpYKV, syntenin/NIYYKV, syntenin/NIpYYKV, syntenin PDZ1 and PDZ2/NIYYKV, syntenin PDZ1 and PDZ2/ NIpYYKV, PHIP PDZ3/GNIpYpYKV, and PHIP PDZ3/GNIpYYKV.

The invention also contemplates antibodies specific for complexes of the invention. The antibodies may be intact monoclonal or polyclonal antibodies, and immunologically active fragments (e.g. a Fab or $(Fab)_2$ fragment), an antibody heavy chain, and antibody light chain, a genetically engineered single chain $F_V$ molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art.

Antibodies specific for the complexes of the invention may be used to detect the complexes in tissues and to determine their tissue distribution. *In vitro* and *in situ* detection methods using the antibodies of the invention may be used to assist in the prognostic and/or diagnostic evaluation of proliferative and/or differentiative disorders associated with a B class ephrin e.g. disorders of the nervous system. Some genetic diseases may include mutations at the binding domain regions of the interacting molecules in the complexes of the invention. Therefore, if a complex of the invention is implicated in a genetic disorder, it may be possible to use PCR to amplify DNA from the binding domains to quickly check if a mutation is contained within one of the domains. Primers can be made corresponding to the flanking regions of the domains and standard sequencing methods can be employed to determine whether a mutation is present.

This method does not require prior chromosome mapping of the affected gene and can save time by obviating sequencing the entire gene encoding a defective protein.

**PHIP Protein**

Broadly stated the present invention contemplates an isolated protein comprising the amino acid sequence shown in Figure 2D and in SEQ. ID. NO.1. The invention contemplates a truncation (i.e. portion) of a protein of the invention, an analog, an allelic or species variation thereof, or a protein having substantial sequence identity with a protein of the invention (i.e. homolog), or a truncation thereof. (Truncations, analogs, allelic or species variations, and homologs are collectively referred to herein as "PHIP Related Proteins").

Truncated proteins may comprise peptides of between 3 and 70 amino acid residues, ranging in size from a tripeptide to a 70 mer polypeptide, preferably 12 to 20 amino acids. In one aspect of the invention, fragments of PHIP protein are provided having an amino acid sequence of at least five consecutive amino acids in Figure 2D and in SEQ. ID. NO. 1, where no amino acid sequence of five or more, six or more, seven or more, or eight or more, consecutive amino acids present in the fragment is present in a protein other than a PHIP Protein. In an embodiment of the invention the fragment is a stretch of amino acid residues of at least 12 to 20 contiguous amino acids from a particular sequence such as a sequence underlined in Figure 2D. The fragments may be immunogenic and preferably are not immunoreactive with antibodies that are immunoreactive to proteins other than a PHIP protein.

In an aspect of the invention, isolated nucleic acids (e.g. SEQ. ID. NO. 33, fragments thereof, complementary and homologous sequences) are provided comprising sequences encoding PHIP protein or PHIP Related Proteins.

The nucleic acids of the invention may be inserted into an appropriate vector, and the vector may contain the necessary elements for the transcription and translation of an inserted coding sequence. Accordingly, vectors may be constructed which comprise a nucleic acid of the invention, and where appropriate one or more transcription and translation elements linked to the nucleic acid molecule.

A vector of the invention can be used to prepare transformed host cells expressing a PHIP protein or a PHIP Related Protein. Therefore, the invention further provides host cells containing a vector of the invention.

The invention also contemplates transgenic non-human mammals whose germ cells and somatic cells contain a recombinant molecule comprising a nucleic acid molecule of the invention in particular one that encodes an analog of a PHIP protein, or a truncation of a PHIP protein.

A PHIP protein or PHIP Related Protein may be obtained as an isolate from natural cell sources, but they are preferably produced by recombinant procedures. In one aspect the invention provides a method for preparing a PHIP protein or a PHIP Related Protein utilizing an isolated nucleic acid molecule of the invention. In an embodiment a method for preparing a PHIP protein or a PHIP Related Protein is provided comprising:

(a) transferring a vector of the invention having a nucleotide sequence encoding a PHIP protein

or PHIP Related Protein, into a host cell;

(b) selecting transformed host cells from untransformed host cells;

(c) culturing a selected transformed host cell under conditions which allow expression of the PHIP protein or PHIP Related Protein and

(d) isolating the PHIP protein or PHIP Related Protein.

The invention further broadly contemplates a recombinant PHIP protein or PHIP Related Protein obtained using a method of the invention.

A PHIP protein or PHIP Related Protein of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins or chimeric proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins.

The invention further contemplates antibodies having specificity against an epitope of a PHIP protein or PHIP Related Protein of the invention. Antibodies may be labeled with a detectable substance and used to detect proteins of the invention in tissues and cells.

The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and accordingly to proteins of the invention. Therefore, the invention also relates to a probe comprising a nucleic acid sequence encoding a protein of the invention, or a part thereof. The probe may be labeled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleic acid molecule of the invention including nucleic acid molecules coding for a protein which displays one or more of the properties of a protein of the invention.

The invention still further provides a method for identifying a substance which binds to a protein of the invention comprising reacting the protein with at least one substance which potentially can bind with the protein, under conditions which permit the binding of the substance and protein; and detecting binding, wherein the detection of binding indicates that the substance binds to the protein. Binding can be detected by assaying for protein-substance complexes, or for activation of the protein. The invention also contemplates methods for identifying substances that bind to other intracellular proteins that interact with a PHIP protein or a PHIP Related Protein. Methods can also be utilized which identify compounds which bind to gene regulatory sequences (e.g. promoter sequences).

Still further the invention provides a method for evaluating a compound for its ability to modulate the biological activity of a PHIP protein or a PHIP Related Protein of the invention. For example, the compound may be a substance that binds to the proteins or a substance that inhibits or enhances the interaction of the protein and a substance that binds to the protein (e.g. a B class ephrin). In an embodiment, the method comprises providing a PHIP protein or a PHIP Related Protein, a substance which binds to the protein, and a test compound under conditions which permit binding of the substance and protein, and detecting binding, wherein the detection of increased or decreased binding relative to binding detected in the absence of the test compound indicates that the test compound modulates the activity of a PHIP protein or a PHIP Related Protein. Binding may be detected by assaying for substance-protein complexes, free substance, and/or free protein, or activation of the protein.

Activation of PHIP or a PHIP Related Protein may be assayed by measuring phosphorylation of the protein, or binding of the protein to cellular proteins, or by assaying for a biological affect on the cell, such as inhibition or stimulation of proliferation, differentiation, or migration.

Compounds which modulate the biological activity of a protein of the invention may also be identified using the methods of the invention by comparing the pattern and level of expression of a PHIP protein or a PHIP Related Protein of the invention in tissues and cells, in the presence, and in the absence of the compounds.

The substances and compounds identified using the methods of the invention may be used to modulate the biological activity of a PHIP protein or a PHIP Related Protein of the invention, and they may be used in the treatment of conditions requiring modulation of the proteins or other molecules that bind to a PHIP protein or a PHIP Related Protein (e.g. a B class ephrin).

**Peptides**

The invention provides peptide molecules that bind to and inhibit the interactions of the molecules in the complexes of the invention. The molecules are derived from the binding domain of a B class ephrin that binds to a PDZ domain containing protein. For example, peptides of the invention include the amino acids YYKV of ephrin B1, B2 or B3 that bind to a PDZ domain containing protein. Other proteins containing these binding domain sequences may be identified with a protein homology search, for example by searching available databases such as GenBank or SwissProt and various search algorithms and/or programs may be used including FASTA, BLAST (available as a part of the GCG sequence analysis package, University of Wisconsin, Madison, Wis.), or ENTREZ (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD).

In accordance with an embodiment of the invention, specific peptides are contemplated that mediate the binding of a B class ephrin and a PDZ domain containing protein. In particular, a peptide of the formula I is provided which interferes with the interaction of a B class ephrin and a PDZ domain containing protein:

$$X\text{-}X^1\text{-}X^2\text{-}K\text{-}V \qquad \text{I}$$

wherein X represents 0 to 70, preferably 0 to 50 amino acids, more preferably 2 to 20 amino acids, and $X^1$ and $X^2$ each represent tyrosine or phosphotyrosine. In specific embodiments, $X^1$ is tyrosine and $X^2$ is phosphotyrosine, $X^1$ is phosphotyrosine and $X^2$ is tyrosine, or $X^1$ and $X^2$ are phosphotyrosine.

In an embodiment of the present invention a peptide of the formula I is provided where X represents NI, GNI, CPHYEKVSGDYGHPVYIVQ(E,D)(M,G)PPQSP(A,P)A (SEQ.ID. NO. 2), GDYGHPVYIVQ(E,D)(M,G)PPQSP(A,P)A (SEQ.ID. NO. 3), PPQSP(A,P)A (SEQ.ID. NO. 4), GPPQSPPNI (SEQ.ID. NO. 32).

Preferred peptides of the invention include the following: YYKV (SEQ ID. NO. 5), GPPQSPPNIpYYKV (SEQ ID. NO. 6), NIpYpYKV (SEQ ID. NO. 7), NIpYYKV (SEQ ID. NO. 8), NIYpYKV (SEQ ID. NO. 9), NIYYKV (SEQ ID. NO. 10), GNIYYKV (SEQ ID. NO. 28), GNIpYpYKV

(SEQ ID. NO. 29), GNIpYYKV (SEQ ID. NO. 30 ), and GNIYpYKV (SEQ ID. NO. 31).

All of the peptides of the invention, as well as molecules substantially homologous, complementary or otherwise functionally or structurally equivalent to these peptides may be used for purposes of the present invention. In addition to full-length peptides of the invention, truncations of the peptides are contemplated in the present invention. Truncated peptides may comprise peptides of about 7 to 10 amino acid residues

The truncated peptides may have an amino group (-NH2), a hydrophobic group (for example, carbobenzoxyl, dansyl, or T-butyloxycarbonyl), an acetyl group, a 9-fluorenylmethoxy-carbonyl(PMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the amino terminal end. The truncated peptides may have a carboxyl group, an amido group, a T-butyloxycarbonyl group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The peptides of the invention may also include analogs of a peptide of the invention and/or truncations of the peptide, which may include, but are not limited to the peptide of the invention containing one or more amino acid insertions, additions, or deletions, or both. Analogs of the peptide of the invention exhibit the activity characteristic of the peptide e.g. interference with the interaction of a B class ephrin and a PDZ domain containing protein, and may further possess additional advantageous features such as increased bioavailability, stability, or reduced host immune recognition. One or more amino acid insertions may be introduced into a peptide of the invention. Amino acid insertions may consist of a single amino acid residue or sequential amino acids.

One or more amino acids, preferably one to five amino acids, may be added to the right or left termini of a peptide of the invention. Deletions may consist of the removal of one or more amino acids, or discrete portions from the peptide sequence. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 7 amino acids.

It is anticipated that if amino acids are inserted or deleted in sequences outside an $NIX^1X^1KV$ sequence that the resulting analog of the peptide will exhibit the activity of a peptide of the invention.

The invention also includes a peptide conjugated with a selected protein, or a selectable marker (see below) to produce fusion proteins.

The peptides of the invention may be prepared using recombinant DNA methods. Accordingly, nucleic acid molecules which encode a peptide of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the peptide. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses so long as the vector is compatible with the host cell used. The expression vectors contain a nucleic acid molecule encoding a peptide of the invention and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be obtained from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San

Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may also be incorporated into the expression vector.

The recombinant expression vectors may also contain a selectable marker gene which facilitates the selection of transformed or transfected host cells. Suitable selectable marker genes are genes encoding proteins such as G418 and hygromycin which confer resistance to certain drugs, β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. The selectable markers may be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes that encode a fusion portion which provides increased expression of the recombinant peptide; increased solubility of the recombinant peptide; and/or aid in the purification of the recombinant peptide by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be inserted in the recombinant peptide to allow separation of the recombinant peptide from the fusion portion after purification of the fusion protein. Examples of fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors may be introduced into host cells to produce a transformant host cell. Transformant host cells include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to include the introduction of nucleic acid (e.g. a vector) into a cell by one of many techniques known in the art. For example, prokaryotic cells can be transformed with nucleic acid by electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells using conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells may be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the peptides of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

The peptides of the invention may be tyrosine phosphorylated using the method described in Reedijk et al. (The EMBO Journal 11(4):1365, 1992). For example, tyrosine phosphorylation may be induced by infecting bacteria harbouring a plasmid containing a nucleotide sequence encoding a peptide of the invention, with a λgt11 bacteriophage encoding the cytoplasmic domain of the Elk tyrosine kinase

as a LacZ-Elk fusion. Bacteria containing the plasmid and bacteriophage as a lysogen are isolated. Following induction of the lysogen, the expressed peptide becomes phosphorylated by the Elk tyrosine kinase.

The peptides of the invention may be synthesized by conventional techniques. For example, the peptides may be synthesized by chemical synthesis using solid phase peptide synthesis. These methods employ either solid or solution phase synthesis methods (see for example, J. M. Stewart, and J.D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford Ill. (1984) and G. Barany and R.B. Merrifield, The Peptides: Analysis Synthesis, Biology editors E. Gross and J. Meienhofer Vol. 2 Academic Press, New York, 1980, pp. 3-254 for solid phase synthesis techniques; and M Bodansky, Principles fo Peptide Synthesis, Springer-Verlag, Berlin 1984, and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biologu, suprs, Vol 1, for classical solution synthesis). By way of example, the peptides may be synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase chemistry with direct incorporation of phosphotyrosine as the N-fluorenylmethoxy-carbonyl-O-dimethylphosphono-L-tyrosine derivative.

N-terminal or C-terminal fusion proteins comprising a peptide of the invention conjugated with other molecules may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of the peptide, and the sequence of a selected protein or selectable marker with a desired biological function. The resultant fusion proteins contain the peptide fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins, glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Cyclic derivatives of the peptides of the invention are also part of the present invention. Cyclization may allow the peptide to assume a more favorable conformation for association with molecules in complexes of the invention. Cyclization may be achieved using techniques known in the art. For example, disulfide bonds may be formed between two appropriately spaced components having free sulfhydryl groups, or an amide bond may be formed between an amino group of one component and a carboxyl group of another component. Cyclization may also be achieved using an azobenzene-containing amino acid as described by Ulysse, L., et al., J. Am. Chem. Soc. 1995, 117, 8466-8467. The side chains of Tyr and Asn may be linked to form cyclic peptides. The components that form the bonds may be side chains of amino acids, non-amino acid components or a combination of the two. In an embodiment of the invention, cyclic peptides are contemplated that have a beta-turn in the right position. Beta-turns may be introduced into the peptides of the invention by adding the amino acids Pro-Gly at the right position.

It may be desirable to produce a cyclic peptide that is more flexible than the cyclic peptides containing peptide bond linkages as described above. A more flexible peptide may be prepared by introducing cysteines at the right and left position of the peptide and forming a disulphide bridge between the two cysteines. The two cysteines are arranged so as not to deform the beta-sheet and turn. The peptide is more flexible as a result of the length of the disulfide linkage and the smaller number of hydrogen bonds in the beta-sheet portion. The relative flexibility of a cyclic peptide can be determined by molecular

dynamics simulations. Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

Peptides that interact with the molecules in a complex of the invention may be developed using a biological expression system. The use of these systems allows the production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate expression vectors. (see Christian et al 1992, J. Mol. Biol. 227:711; Devlin et al, 1990 Science 249:404; Cwirla et al 1990, Proc. Natl. Acad, Sci. USA, 87:6378). Libraries may also be constructed by concurrent synthesis of overlapping peptides (see U.S. Pat. No. 4,708,871).

Peptides of the invention may be used to identify lead compounds for drug development. The structure of the peptides described herein can be readily determined by a number of methods such as NMR and X-ray crystallography. A comparison of the structures of peptides similar in sequence, but differing in the biological activities they elicit in target molecules can provide information about the structure-activity relationship of the target. Information obtained from the examination of structure-activity relationships can be used to design either modified peptides, or other small molecules or lead compounds which can be tested for predicted properties as related to the target molecule. The activity of the lead compounds can be evaluated using assays similar to those described herein.

Information about structure-activity relationships may also be obtained from co-crystallization studies. In these studies, a peptide with a desired activity is crystallized in association with a target molecule, and the X-ray structure of the complex is determined. The structure can then be compared to the structure of the target molecule in its native state, and information from such a comparison may be used to design compounds expected to possess desired activities.

The peptides of the invention may be converted into pharmaceutical salts by reacting with inorganic acids such as hydrochloric acid, sulfuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benezenesulfonic acid, and toluenesulfonic acids.

The peptides of the invention may be used to prepare antibodies. Conventional methods can be

used to prepare the antibodies.

The peptides and antibodies specific for the peptides of the invention may be labelled using conventional methods with various enzymes, fluorescent materials, luminescent materials and radioactive materials. Suitable enzymes, fluorescent materials, luminescent materials, and radioactive material are well known to the skilled artisan. Antibodies and labeled antibodies specific for the peptides of the invention may be used to screen for proteins containing PDZ domain binding sites.

Computer modelling techniques known in the art may also be used to observe the interaction of a peptide of the invention, and truncations and analogs thereof with a molecule in a complex of the invention e.g. PDZ domain containing protein (for example, Homology Insight II and Discovery available from BioSym/Molecular Simulations, San Diego, California, U.S.A.). If computer modelling indicates a strong interaction, the peptide can be synthesized and tested for its ability to interfere with the binding of the molecules of a complex discussed herein.

**Methods for Identifying or Evaluating Substances/Compounds**

The methods described herein are designed to identify substances and compounds that modulate the activity of a complex of the invention thus potentially affecting cellular processes associated with B class ephrins and/or PDZ domain containing proteins. Novel substances are therefore contemplated that bind to molecules in the complexes, or bind to other proteins that interact with the molecules, to compounds that interfere with, or enhance the interaction of the molecules in a complex, or other proteins that interact with the molecules.

The substances and compounds identified using the methods of the invention include but are not limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide libraries and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), antibodies [e.g. polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (e.g. Fab, $F(ab)_2$, and Fab expression library fragments, and epitope-binding fragments thereof)], and small organic or inorganic molecules. The substance or compound may be an endogenous physiological compound or it may be a natural or synthetic compound.

Substances which modulate the activity of a complex of the invention can be identified based on their ability to bind to a molecule in the complex. Therefore, the invention also provides methods for identifying novel substances which bind molecules in the complex. Substances identified using the methods of the invention may be isolated, cloned and sequenced using conventional techniques.

Novel substances which can bind with a molecule in a complex of the invention may be identified by reacting one of the molecules with a test substance which potentially binds to the molecule, under conditions which permit binding of the molecule and test substance, and detecting binding. Binding may be detected by assaying for substance-molecule conjugates, for free substance, or for non-complexed molecules, or activation of the molecule. Conditions which permit the formation of substance-molecule conjugates may be selected having regard to factors such as the nature and amounts of the substance and

the molecule.

The substance-molecule conjugate, free substance or non-complexed molecules may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against the molecule or the substance, or labeled molecule, or a labeled substance may be utilized. The antibodies, proteins, or substances may be labeled with a detectable substance as described above.

Activation may be assayed by measuring phosphorylation of a molecule, binding of receptors or cellular proteins to a molecule, or in a cellular assay, by assaying for a biological affect on the cell, such as inhibition or stimulation of proliferation, differentiation or migration.

A molecule, or complex of the invention, or the substance used in the method of the invention may be insolubilized. For example, a molecule, or substance may be bound to a suitable carrier such as agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized protein or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The invention also contemplates a method for evaluating a compound for its ability to modulate the biological activity of a complex of the invention, by assaying for an agonist or antagonist of the binding of the molecules in the complex. The basic method for evaluating if a compound is an agonist or antagonist of the binding of molecules in a complex of the invention, is to prepare a reaction mixture containing the molecules and the test compound under conditions which permit the molecules to bind and form a complex. The test compound may be initially added to the mixture, or may be added subsequent to the addition of molecules. Control reaction mixtures without the test compound or with a placebo are also prepared. The formation of complexes is detected and the formation of complexes in the control reaction but not in the reaction mixture indicates that the test compound interferes with the interaction of the molecules. Increased complex formation relative to a control reaction indicates that the test compound enhances the interaction of the molecules. The reactions may be carried out in the liquid phase or the molecules, or test compound may be immobilized as described herein.

It will be understood that the agonists and antagonists that can be assayed using the methods of the invention may act on one or more of the binding sites on the interacting molecules in the complex including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of molecules in a complex of the invention. Thus, the invention may be used to assay for a compound that competes for the same binding site of a molecule in a complex of the invention.

The invention also contemplates methods for identifying novel compounds that bind to proteins that interact with a molecule of a complex of the invention. Protein-protein interactions may be identified using conventional methods such as co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Methods may also be employed that result in the simultaneous identification of genes which encode proteins interacting with a molecule. These methods include probing expression libraries with labeled molecules. Additionally, x-ray crystallographic studies may be used as a means of evaluating interactions with substances and molecules. For example, purified recombinant molecules in a complex of the invention when crystallized in a suitable form are amenable to detection of intra-molecular interactions by x-ray crystallography. Spectroscopy may also be used to detect interactions and in particular, a quadrupole/time-of-flight hybrid instrument (QqTOF) may be used.

Two-hybrid systems may also be used to detect protein interactions *in vivo*. Generally, plasmids are constructed that encode two hybrid proteins. A first hybrid protein consists of the DNA-binding domain of a transcription activator protein fused to a molecule in a complex of the invention, and the second hybrid protein consists of the transcription activator protein's activator domain fused to an unkown protein encoded by a cDNA which has been recombined into the plasmid as part of a cDNA library. The plasmids are transformed into a strain of yeast (e.g. *S. cerevisiae*) that contains a reporter gene (e.g. lacZ, luciferase, alkaline phosphatase, horseradish peroxidase) whose regulatory region contains the transcription activator's binding site. The hybrid proteins alone cannot activate the transcription of the reporter gene. However, interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

It will be appreciated that fusion proteins and recombinant fusion proteins may be used in the above-described methods. It will also be appreciated that the complexes of the invention may be reconstituted *in vitro* using recombinant molecules and the effect of a test substance may be evaluated in the reconstituted system.

The reagents suitable for applying the methods of the invention to evaluate substances and compounds may be packaged into convenient kits providing the necessary materials packaged into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

<u>Compositions and Treatments</u>

The complexes, peptides, and antibodies of the invention, and substances and compounds identified using the methods of the invention may be used to modulate cellular processes such as proliferation, growth, and/or differentiation of cells associated with B class ephrins and/or PDZ domain containing proteins (in particular axonogenesis, nerve cell interactions and regeneration of the nervous system). Therefore they may be used to treat conditions in a subject in which the compounds or substances are introduced. Thus, the substances may be used for the treatment of disorders associated with a B class ephrin such as disorders of the nervous system including neurodegenerative diseases and cases of nerve injury.

Accordingly, the complexes, peptides, substances, antibodies, and compounds may be formulated

into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a "therapeutically active amount" of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions that may inactivate the compound.

The compositions described herein can be prepared by <u>per se</u> known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances or compounds in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The activity of the complexes, substances, compounds, antibodies, and compositions of the invention may be confirmed in animal experimental model systems.

The invention also provides methods for studying the function of a complex of the invention. Cells, tissues, and non-human animals lacking in the complexes or partially lacking in molecules in the complexes may be developed using recombinant expression vectors of the invention having specific deletion or insertion mutations in the molecules. A recombinant expression vector may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create complex deficient cells, tissues or animals. Null alleles may be generated in cells and may then be used to generate transgenic non-human animals.

The following non-limiting example is illustrative of the present invention:

**Example**

**EXPERIMENTAL PROCEDURES**

**Peptide synthesis**

The B ephrin C-terminal peptide probe of sequence biotin-Aca-GPPQSPPNIpYYKV (SEQ. ID. NO.6), related peptides NIpYpYKV (SEQ. ID. NO. 7), NIpYYKV (SEQ. ID. NO. 8), NIYpYKV (SEQ. ID. NO. 9), NIYYKV (SEQ. ID. NO. 10), and DHQpYpYND (SEQ. ID. NO. 11), were synthesized as described previously (29).

**Isolation of PDZ domain-encoding cDNA clones**

A λEXlox 10.5 day mouse embryo expression library (Novagen) was plated at an initial density of 10,000 plaque-forming units/15 cm petri plate. Library screening was performed using a biotinylated peptide probe conjugated to streptavidin-alkaline phosphatase following a procedure similar to that described by Sparks et al. (30). To isolate more coding sequence for PHIP, an *EcoRI/Pst I* fragment of PHIP cDNA (encoding amino acid residues 462-602) was radiolabelled with [$\alpha$-$^{32}$P]dCTP and used to screen the λEXlox 10.5 day mouse embryo library. The DNA sequencing of positive clones was carried out using the ALF automated DNA sequencer (Amersham Pharmacia Biotech).

**Antibodies, constructs and mutagenesis**

Anti-ligand antibodies (Santa Cruz) were raised against residues 329-346 of hEphrin B1. Anti-FLAG M2 monoclonal antibodies were purchased from Eastman Kodak Company. The expression construct of ephrin B1 cDNA in vector pJFE14 has been described (18). Full-length syntenin cDNA was subcloned in frame into the mammalian expression vector pFLAG CMV2 (Eastman Kodak) using standard cloning procedures. For GST fusion constructs, cDNA sequences of syntenin (full length: residues 1-299; PDZ 1+2: residues 101-299; PDZ1: residues 101-211; PDZ2: residues 172-299) were cloned into pGEX4T2 (Amersham Pharmacia Biotech). FAP-1 (Fas associated phosphatase) PDZ3 and FAP-1 PDZ5 constructs have been described (1). The ephrin B1 Val deletion mutation was constructed by the removal of nucleotides coding for the C-terminal V346 using a PCR-mediated protocol. The *PpuMI/EcoRI* PCR fragment carrying the mutated region was subcloned into the full-length ephrin B1 cDNA in pJFE14. This mutation and all fusion constructs were confirmed by sequencing of both strands of the affected region.

**Immunoprecipitation and Western blot analysis**

Cos-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Transient transfections were performed using Lipofectin reagent and Opti-MEM medium (Life Technologies Inc.) as outlined by the manufacturer. To reduce phosphorylation of ephrin B1 by binding to endogenously expressed EphB receptors or by stimulation with serum growth factors, transfected cells were transferred from 10 cm to 15 cm plates 24 h after transfection and serum starved in DMEM 0.5% FBS 12 h prior to cell lysis. Transfected cells were rinsed once in PBSA and lysed in PLC lysis buffer (5) with 10 μg/ml aprotonin, 10 μg/ml leupeptin, 1 mM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride added. Immunoprecipitations were performed for 1 h at 4°C using 1 μg anti-ephrin B1 antibody or 1 μg anti-IL-3 receptor α antibody with protein A-sepharose. GST mixing experiments were carried

out by 1 h incubation at 4 °C of lysate with 5-10 μg of fusion protein immobilized on glutathione sepharose. For the peptide competition experiments, peptides were included in the incubation with the GST fusion proteins at a final concentration of 100 μM. Beads for both immunoprecipitations and GST mixing experiments were washed 2-3 times in HNTG buffer (5). Proteins were separated by 10% SDS-PAGE, transferred to Immobilon-P membrane (Millipore) and immunoblotted with the appropriate antibody. Blots were developed by Enhanced Chemiluminescence (Pierce).

**Fluorescence Polarization Analysis**

Binding constant determination and peptide competition studies were carried out using fluorescence polarization on a Beacon 2000 Fluorescence Polarization System (Pan Vera, WI) equipped with a 100-μl sample chamber. Fluorescein-labeled probes were prepared through reaction of B ephrin C-terminal peptides with 5-(and-6-)-carboxyfluorescein, succinimidyl ester (Molecular Probes, OR) and purified by reverse-phase HPLC. The authenticity of the fluorescein-labelledpeptides were confirmed by mass spectroscopy. In the binding studies, the fluorescein-labelledpeptide probe was dissolved in 20 mM phosphate pH 7.0, 100 mM NaCl, and 2 mM DTT to a concentration of 25 nM and a known quantity of GST- fusion protein added. The reaction mixtures were allowed to stand for 10 min at room temperature prior to each measurement. All fluorescence polarization measurements were conducted at 22°C.

<u>**RESULTS**</u>

**Identification of potential binding partners for the putative PDZ binding site of B ephrins**

As one approach towards identifying proteins that interact with the cytoplasmic tails of B-type ephrins, the C-terminal regions of the transmembrane ephrins were initially examined for conserved peptide motifs that might bind modular domains of intracellular signaling proteins. The extreme carboxy terminus of the three known B ephrins has a conserved sequence reminiscent of known or predicted binding sites for PDZ domains (Fig. 1). Two strategies were employed to identify PDZ domain-containing proteins with the potential to recognize the B ephrins. Firstly, comparison of the known binding specificities of PDZ domains, predicted through the use of an oriented peptide library technique, revealed the fifth PDZ domain of the cytoplasmic tyrosine phosphatase FAP-1 (Fas-associated phosphatase) as a possible ephrin B binding partner (Fig. 2A). FAP-1 (also known as PTP-bas and PTP-L1) has at least six PDZ domains, an element related to the Band 4.1 cytoskeletal polypeptide, and a C-terminal tyrosine phosphatase domain (31-33). The fifth PDZ domain binds *in vitro* to peptides with the consensus E-(I/Y/V)-Y-(Y/K)-(V/K/I), which closely matches the conserved C-terminus of B-type ephrins (YYKV) (1).

A more direct approach to isolate ephrin B-binding proteins was undertaken by screening a cDNA expression library from a day 10.5 mouse embryo with a peptide probe based on the putative PDZ domain binding site of ephrin B3. The probe was a biotinylated peptide, biotin-Aca-GPPQSPPNIpYYKV (SEQ. ID. No. 6), conjugated to streptavidin-alkaline phosphatase. Although this peptide contained a phosphotyrosine residue at the –3 position relative to the C-terminal valine, it was anticipitated that the alkaline phosphatase used in the screen would at least partially dephosphorylate the probe, allowing

detection of both tyrosine phosphorylation dependent and independent binding. The screening of approximately 500,000 cDNA clones yielded four distinct cDNA products that bound to the ephrin B3 C-terminal peptide, of which three were subsequently found to contain PDZ domains upon sequence analysis (Fig. 2B and 2C). One of these cDNAs encodes a portion of the adaptor protein GRIP, from the sixth PDZ domain to the carboxy terminus (amino acid residues 642-1112). GRIP is an ~180 kDa protein composed of seven PDZ domains, originally identified by its ability to bind the C-terminus of AMPA receptors through PDZ domains 4 and 5 (34). A second cDNA isolated by this approach contained the entire coding sequence for the PDZ domain-containing protein syntenin. Syntenin was first reported as a transcript down-regulated during melanoma differentiation (termed Mda-9) and subsequently shown to interact via its two PDZ domains with the C-terminus of the transmembrane syndecan proteins (35,36). A third clone identified in this screen was a partial cDNA encoding the carboxy-terminal fragment of a novel PDZ domain-containing protein (termed PHIP for ephrin interacting protein). Analysis of the sequence of the PHIP cDNA fragment revealed the presence of two adjacent PDZ domains followed by a 50 amino acid C-terminal stretch. The PHIP cDNA fragment was subsequently used as a probe to isolate a transcript from a day 10.5 mouse embryo library. The predicted sequence of PHIP indicates that it encodes a total of three PDZ domains and is closely related to PAR-3, a *C. elegans* protein involved in regulating polarity of the early embryo (Fig. 2D) (37). Of these candidates, FAP-1 PDZ5 and syntenin were further investigated for their binding to B ephrins.

**Syntenin and FAP-1 PDZ5 bind ephrin B1 *in vitro***

To determine if either syntenin or FAP-1 could interact with ephrin B1 *in vitro*, GST-fusions containing the fifth PDZ domain of FAP-1 or full-length syntenin were incubated with lysates of ephrin B1-transfected Cos-1 cells. Recovery of these immobilized GST fusion proteins and immuno-blotting of associated proteins with anti-ephrin B1 antibody revealed that both FAP-1 PDZ5 and full-length syntenin were able to bind intact ephrin B1 (Fig. 3A and 3C). The region of syntenin required for binding to ephrin B1 was mapped using GST fusions containing defined fragments of the syntenin protein. The minimal sequence necessary for a strong interaction included both PDZ domains of syntenin but not the amino-terminal third of the protein (Fig. 3D). Interestingly, both PDZ domains of syntenin are also required for binding to the C-terminal sequence of syndecans, suggesting that the involvment of two PDZ domains in the binding of a single target site may be a common feature of syntenin interactions (36). While the syntenin PDZ1 domain alone was unable to associate with ephrin B1, the second PDZ domain of syntenin alone, exhibited a very weak interaction.

In these experiments, neither GST alone nor a GST fusion with the third FAP-1 PDZ domain showed detectable binding to ephrin B1. The identity of the ~50 kD band recognized by GST-FAP-1 PDZ3 is not known but its apparent size does not correlate with any of the three known B ephrins. Consistent with this finding, the binding specificity of FAP-1 PDZ3, as previously determined using an oriented peptide library, is significantly different from that of FAP-1 PDZ5, with a preference towards target

sequences such as the QSLV-COOH motif in the Fas antigen (1,33). The inability of the of FAP-1 PDZ3 domain to bind ephrin B1 indicates a degree of specificity in recognition of ephrin B1 by PDZ domains.

A hallmark of many PDZ domain binding sites is a requirement for a C-terminal hydrophobic residue that contacts the PDZ domain through its side chain and C-terminal carboxylate group (1, 38,39). The involvement of the C-terminal Val of ephrin B1 in specific binding to syntenin and FAP-1 PDZ5 was initially evaluated by expressing a deletion mutant of ephrin B1 lacking the terminal Val residue in Cos-1 cells. Removal of the C-terminal Val from full-length ephrin B1 abrogated its binding to both syntenin and FAP-1 PDZ5 GST fusion proteins (Fig. 3B and 3C).

As an alternative approach towards investigating the specificity of ephrin B1 interactions with PDZ domain proteins, a specific peptide modeled on the C-terminus of B-type ephrins was employed in competition experiments. For this purpose, lysates of ephrin B1-transfected cells were incubated with either GST-syntenin or GST-FAP-1 PDZ5 in the presence or absence of a peptide corresponding in sequence to the C-terminal six residues of B ephrins. The peptide successfully blocked syntenin and FAP-1 PDZ5 binding at a peptide concentration of 100 μM (Fig. 4A and 4B). The addition of the unrelated peptide, DHQpYpYND (SEQ. ID. NO. 11), did not decrease binding, indicating the specificity of the peptide competition (Fig. 4A).

**FAP-1 PDZ5 and syntenin display differential binding to phosphopeptides**

Binding of B ephrins to their cognate Eph B receptors, expression of an activated Src tyrosine kinase or treatment of ligand-expressing cells with PDGF results in tyrosine phosphorylation of residues in the ephrin cytoplasmic domain (26,27). Preliminary evidence based on specific substitutions of the Tyr residues in the ephrin B1 tail indicates that the two tyrosines at the –2 and –3 positions within the PDZ domain binding site are among the phosphorylation sites. To investigate whether tyrosine phosphorylation of these residues might affect PDZ domain binding, the C-terminal peptide used for the peptide competition described above was also synthesized such that either one or both of the –2 and –3 tyrosine residues were phosphorylated. The phosphorylated and unphosphorylated peptides were labeled with fluorescein and employed in fluorescence polarization experiments to obtain quantitative measurements of their affinities for FAP-1 and syntenin PDZ domains.

The GST-FAP-1 PDZ5 bound to a fluorescein-labeledNIYYKV (SEQ. ID. NO. 10) peptide with an affinity of 9.9 ± 1.0 μM, while GST-FAP-1 PDZ3 binding was much weaker (65.0 ± 9.6 μM) (Fig. 5A). This is consistent with the GST mixing experiments that indicated FAP-1 PDZ3 does not interact stably with ephrin B1. Similar results were obtained when binding to the three different phosphorylated peptides was investigated, indicating that alternative tyrosine phosphorylation states of the B ephrin C-terminal sequence had little effect on binding to GST-FAP-1 PDZ5. Similar binding affinity values of 6.8 ± 0.8 μM, 15.4± 3.4 μM and 8.4 ± 2.5 μM were obtained for the NIpYYKV, NIYpYKV and NIpYpYKV (SEQ. ID. NO. 8, 9, and 7 respectively) peptides, respectively.

Fluorescence polarization experiments measuring GST-syntenin fusion protein binding to fluorescein-labeledNIYYKV (SEQ. ID. NO. 10) and NIpYYKV (SEQ. ID. NO. 8) peptides yielded nearly

identical binding curves (Fig. 5B). Affinity values of 17.7 ± 1.2 μM and 15.4 ± 0.5 μM were obtained, indicating that phosphorylation at the –3 position tyrosine does not significantly affect the PDZ-domain interaction. However, the GST-syntenin fusion protein bound the pYpYKV (SEQ. ID. NO. 12) peptide with a much lower affinity of 151.0 ± 20.9 μM, indicating that phosphorylation at the -2 Tyr can have a detrimental effect on binding to syntenin. A similar low affinity interaction was observed for the YpYKV peptide.

**Ephrin B1 and syntenin can associate in cells**

The possibility that B-type ephrins may interact with PDZ domain proteins *in vivo* was pursued by assaying whether ephrin B1 and syntenin associate when co-expressed in Cos-1 cells. In cells co-transfected with ephrin B1 and syntenin (tagged at its N-terminus with a FLAG epitope) immunoprecipitation of ephrin B1 specifically co-precipitated syntenin (Fig. 6). Precipitation with protein A sepharose alone or with an arbitrarily chosen antibody did not yield detectable syntenin, indicating that the interaction is specific. Further, co-immunoprecipitation experiments with the ephrin B1 Val deletion mutant, which fails to interact with PDZ domains *in vitro*, showed that ephrin B1 lacking the C-terminal Val did not detectably associate with syntenin (Fig. 6). While the truncated protein could be successfully immunoprecipitated by antibodies against ephrin B1, syntenin could not be co-immunoprecipitated with the mutant protein. These results demonstrate that ephrin B1 and syntenin can associate in cells, and show that an intact PDZ domain binding site in ephrin B1 is necessary for its interaction with syntenin *in vivo*.

**DISCUSSION**

In an effort to identify components of the cytoplasmic domain that may contribute to ephrin B function, it was demonstrated that the C-terminal residues of B ephrins constitute a binding site for PDZ domains, a class of protein module known to mediate specific protein-protein interactions. Several lines of evidence indicate that the C-terminal YYKV sequence, conserved among all 3 known B ephrins, represents a PDZ domain binding site. Firstly, a biotinylated peptide probe with a sequence corresponding to the C-terminal residues of ephrin B3 identified cDNAs coding for the known PDZ domain-containing proteins syntenin and GRIP, as well as a cDNA for PHIP, a novel PDZ domain-containing protein. In addition, a fourth PDZ-containing protein, FAP-1, was identified as a binding candidate based initially on the predicted binding specificity of its fifth PDZ domain.

Secondly, *in vitro* studies with syntenin and FAP-1 have demonstrated specific interactions of the PDZ domains of these proteins with the C-terminus of ephrin B1. The finding that the C-terminal Val residue of ephrin B1 is absolutely required for these interactions indicates that binding occurs in a manner characteristic of other PDZ domain interactions with C-terminal target sequences. Similar results were also obtained from *in vitro* binding experiments with ephrin B2, suggesting that PDZ domain interactions may be common to all B ephrins. *In vitro* experiments were also performed with separate GST fusions of GRIP PDZ6 and GRIP PDZ7. Interactions with ephrin B1 or with the fluorescent GNIYYKV (SEQ. ID. NO. 13) peptide were not detected in GST-mixing and fluorescence polarization experiments. Binding to ephrin B1 may require both PDZ 6 and PDZ 7 of GRIP in a fashion reminiscent of the requirement of both

syntenin PDZ domains for binding. Lastly, it was demonstrated that B ephrin-PDZ domain interactions can occur *in vivo*, since syntenin can be successfully co-immunoprecipitated with full-length ephrin B1 but not with ephrin B1 truncated in its PDZ domain target site.

The effect of the phosphorylation state of two adjacent tyrosines at positions –2 and –3 relative to the C-terminal Val of the PDZ domain target site was examined using a fluorescence polarization assay. Structural studies of PDZ domains have suggested that interactions between PDZ domains and residues at the –2 and –3 positions of the C-terminal target site confer binding specificity (38-40). In one case, modification of residues at these positions by serine phosphorylation has been reported to regulate PDZ domain binding. The specific association between the second PDZ domain of PSD-95 and the inward rectifier potassium ($K^+$) channel Kir2.3 is disrupted by protein kinase A mediated phosphorylation of a key serine residue at the –2 position from the C-terminus of Kir2.3 (41). The results with B class ephrins and the PDZ domain proteins FAP-1 and syntenin suggest that the phosphorylation of residues within the PDZ domain binding site has different effects on different PDZ domains. The results with FAP-1 PDZ5 suggest that the PDZ domain residues which contact the tyrosines in the binding site of B ephrins are able to accommodate the addition of two phosphate groups. This is consistent with observations that the single PDZ domain of AF-6 binds an unphosphorylated peptide with the consensus target sequence AYYV (SEQ. ID. NO. 14) and a corresponding peptide phosphorylated at the –2 Tyr residue with approximately equal affinity. In contrast, GST-syntenin exhibited significantly decreased binding to peptides phosphorylated at the –2 residue of the PDZ domain binding site. These data indicate one mechanism through which tyrosine phosphorylation of ephrin B1 may regulate interactions with modular cytoplasmic proteins. Possible roles for PDZ domain-ephrin B associations can be proposed based on known functions of PDZ domains. Several examples have highlighted the importance of PDZ domain interactions in the proper localization and clustering of transmembrane proteins (42,43). For instance, the positioning of NMDA receptors and $K^+$ channels at post-synaptic termini is likely dependent on specific interactions of these receptors with PDZ domain-containing proteins (34, 44-47). In *Drosophila* larvae, null mutations of the gene encoding the PDZ protein discs-large result in mislocalization of the Shaker $K^+$ channel (48). Clustering of Shaker $K^+$ channels via PDZ domain interactions has also been demonstrated in COS7 cells co-expressing the channel with either of its binding partners, PSD-95 or chapsyn 110 (49).

A requirement for correct localization and clustering figures prominently in the proposed functions of B class ephrins. Since ephrin B-EphB interactions involve direct cell-cell contact, ephrins must be present at sites of contact with receptor-expressing cells. This localization may be mediated by PDZ domain associations with the C-terminus of B ephrins. In this regard, it is of interest that PHIP is a close relative of PAR-3, a *C. elegans* protein that regulates asymmetry and polarity in the early embryo. It is possible that PHIP has a similar function in mammalian cells in controlling the asymmetric distribution of proteins with PDZ domain-binding motifs. Studies involving soluble forms of the extra-cellular domain of ephrins have revealed a requirement for ligand clustering in receptor activation. Whereas treatment of receptor-expressing cells with soluble versions of the ligands does not result in

receptor activation and subsequent autophosphorylation, artificial aggregation of soluble ephrins by clustering antibodies allows activation of the receptor (18). Since co-culturing of ephrin-expressing cells with cells expressing Eph receptors leads to receptor activation, membrane-bound ligands must also become clustered in some manner. Furthermore, recent studies in a renal endothelial cell system have indicated that the state of ephrin B1 oligomerization is important in determining alternative receptor signaling complexes as well as attachment and assembly responses in the receptor-bearing cell (50). Although binding of both ligand dimers and higher order oligomers cause receptor autophosphorylation, only tetrameric forms of the ligand were able to induce the attachment response and stimulate the recruitment of low molecular weight phosphotyrosine phosphatase to the activated receptor. Given the known role of PDZ domains in the clustering of transmembrane proteins, PDZ domain interactions with ephrin B1 may play a role in the presentation of the ligand in the correct oligomeric form to elicit specific responses in the receptor-expressing cell.

Another role ascribed to PDZ domain-containing proteins is to act as a scaffold to organize signaling complexes. This is well illustrated by the function of the protein InaD in the photo-transduction pathway of the *Drosophila* compound eye. Key components of this cascade, including the transient receptor potential (TRP) calcium channel, the eye form of protein kinase C and phospholipase C-β are bound by the PDZ domains of InaD to form a compartmentalized signalling complex (51,52). Mutations in specific InaD PDZ domains that abolish binding result in defects in the kinetics of the phototransduction cascade. In the case of B ephrins, genetic evidence along with biochemical studies indicating that tyrosine residues in the intracellular domain become phosphorylated upon receptor binding or PDGF treatment has led to the hypothesis that the cytoplasmic tail of B ephrins may have an intrinsic signaling function (2,6,26,27). The phosphorylated tyrosine residues represent potential docking sites for proteins with phosphotyrosine recognition modules such as SH2 or PTB domains. Downstream components of this possible phosphotyrosine-dependent signaling pathway may be assembled around a PDZ domain-containing protein in a manner similar to the InaD complex. Furthermore, the PDZ domain-containing protein PSD-95 which associates with glutamate receptors and $K^+$ channels also interacts through its PDZ domains with neuronal nitric oxide synthase and a Ras GTPase activating protein (p135 SynGAP) (53,54). PDZ domain-containing proteins may thereby serve as adaptors to directly activate signaling pathways. In this context, it is of interest that phosphorylation of the Tyr residues in the C-terminal ephrin B1 motif may regulate interactions with PDZ domains, as suggested by the results with syntenin.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. All modifications coming within the scope of the following claims are claimed.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

**Detailed Description of the Drawings**

Fig. 1. **Amino acid sequence of the cytoplasmic domains of the human B ephrins.** Conserved residues among the three B ephrins are highlighted. Asterisks mark conserved tyrosines that are potential sites of phosphorylation. The potential PDZ domain binding site is underlined.

Fig. 2A-D. **Identification of PDZ domain-containing candidates for ephrin B binding.** Fig. 2*A*, The preferred binding sequence of FAP-1 PDZ5 is shown below a schematic representation of the entire FAP-1 protein tyrosine phosphatase. FAP-1 PDZ5 domain specificity was deduced from an oriented peptide library technique (1). Residues within the optimal binding sequence that match the C-terminal sequence of B ephrins are indicated in bold. The organization of the PDZ domains of FAP-1 shown in this figure follows the numbering described by Sato *et al.* (33). Fig.2*B*, Diagrammatic representations of the PDZ domain-containing proteins identified through an expression screen with a biotinylated peptide probe of eprhin B3 C-terminal sequence. The brackets mark the portions of the protein encoded by the cDNAs isolated from the screen. PDZ domains are represented by grey boxes. Fig. 2*C*, Amino acid sequence alignment of FAP-1 PDZ5 and of the PDZ domains isolated in the expression screen. The numbering of the PDZ domains is as shown in Fig. 2B. Conserved residues are highlighted. The alignment was performed with the ClustalW program (55). Fig.2*D*, Amino acid sequence alignment of PHIP and PAR-3. Conserved residues are highlighted and the PDZ domains are underlined. The alignment was performed with the Genestream Align program.

Fig. 3A-C. **FAP-1 PDZ5 and syntenin bind specifically to ephrin B1 in GST-mixes.** Cos-1 cells were transiently transfected with either wild-type ephrin B1 (W.T.) or the ephrin B1 Val deletion (Val Δ) or were untransfected. Cell lysates were incubated with the GST fusion proteins as indicated and analyzed by immunoblotting with anti-ephrin B1 antibody. Immunoprecipitated ephrin B1 or ephrin B1 Val Δ were included as a positive control. Fig. 3*A* and Fig, 3*B*, GST-mixes with fusion proteins of FAP-1. *C* and *D*, GST-mixes with fusion proteins of syntenin.

Fig. 4A and 4B. **FAP-1 PDZ5 and syntenin binding to ephrin B1 can be blocked by addition of peptides corresponding to the C-terminal sequence of B ephrins.** Peptides of the indicated sequence were included at a concentration of 100μM in incubations of GST fusion proteins with lysates of Cos-1 cells transfected with ephrin B1. Associated proteins were separated on a 10% polyacrylamide/SDS gel and analyzed by immunoblotting with antibodies against ephrin B1. Fig. 4*A*, Competition of FAP-1 PDZ5 binding to ephrin B1 using the indicated peptides. A peptide of sequence DHQpYpYND was added at a concentration of 100 μM as a negative control. Immunoprecipitation of ephrin B1 was included as a positive control. Fig. 4*B*, Peptide competition of the binding of full-length syntenin to ephrin B1.

Fig. 5A and 5B. **Fluorescence polarization analysis of GST-FAP-1 PDZ3, GST-FAP-1 PDZ5 and GST-syntenin binding to Fluorescein-labelled peptides corresponding to the C-terminus of ephrin B1.** Fig. 5 *A*, Solutions containing the indicated final concentration of GST-FAP-1 PDZ3 (O) or GST-FAP-1 PDZ5 ( ) fusion protein in mixtures containing 25 nM fluorescein-labelled NIYYKV peptide

probe, 20 mM phosphate pH 7.0, 100 mM NaCl , and 2 mM dithiothreitol (DTT) were monitored for fluorescence polarization at 22°C. The GST-FAP-1 PDZ5 fusion protein was also measured for binding to the phosphorylated peptides, NIpYYKV (▼), NiYpYKV (▲) and NIpYpYKV (■). The fluorescence polarization values obtained for the peptide in absence of added GST-fusion protein has been subtracted from the polarization values displayed. Fig. *5B*, A Binding of a GST fusion of full-length syntenin to the NIYYKV (●), NIpYYKV (▼), and NIpYpYKV (■) peptides as measured by fluorescence polarization.

Fig. 6. **Co-immunoprecipitation of syntenin-FLAG with ephrin B1.** Cos-1 cells were co-transfected with either ephrin B1 and syntenin-FLAG or with the ephrin B1 Val deletion and syntenin-FLAG as indicated. Cell lysates were immunoprecipitated with antibodies against ephrin B1 or IL-3 receptor α or were treated with protein A sepharose only. Immunocomplexes were subjected to SDS-PAGE (10%) and blotted with anti-FLAG antibodies.

Fig. 7. **Fluorescence polarization analysis of GST-PHIP PDZ3 binding to Fluorescein-labelled peptides corresponding to the C-terminus of ephrin B1.** Solutions containing the indicated final concentration of GST-PHIP PDZ3 fusion protein in mixtures containing 25 nM fluorescein-labelled peptide probe, 20 mM phosphate pH 7.0, 100 mM NaCl , and 2 mM DTT were monitored for fluorescence polarization at 22°C. The GST-PHIP PDZ3 fusion protein was measured for binding to the phosphorylated peptides, NIpYYKV (▼), NiYpYKV (▲) and NIpYpYKV (■) and the unphosphorylated NIYYKV peptide (●). The fluorescence polarization values obtained for the peptide in absence of added GST-fusion protein has been subtracted from the polarization values displayed.

Fig. 8 **PHIP PDZ3 binds specifically to V-Src phosphorylated ephrin B1 in GST-mixes.** COS-1 cells were transiently co-transfected with V-Src and either wild-type ephrin B1 or the ephrin B1 Val deletion (VΔ) or were transfected with either wild-type ephrin B1 or ephrin B1 Val deletion alone. Cell lysates were incubated with the GST fusion proteins as indicated and analyzed by immunoblotting with anti-phosphotyrosine antibody. Immunoprecipitated ephrin B1 was included as a positive control.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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We Claim:

1. An isolated complex comprising a B class ephrin and a PDZ domain containing protein.

2. An isolated complex as claimed in claim 1 wherein the B class ephrin is ephrin B1 or ephrin B3.

3. An isolated complex as claimed in claim 1 or 2 wherein the PDZ domain containing protein is GRIP, GRIP PDZ6 and PDZ 7 of SEQ.ID.NO. 22 and 23, FAP-1 PDZ of SEQ. ID. NO. 21, amino acids residues 1 to 299 of syntenin, syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27, PHIP PDZ2 of SEQ. ID. NO. 24; and PHIP PDZ3 of SEQ. ID. NO. 25.

4. An isolated complex as claimed in claim 3 which is ephrin B3/GRIP; ephrin B3/GRIP PDZ6 and PDZ 7 of SEQ.ID.NO. 22 and 23; ephrin B1/FAP-1 PDZ of SEQ. ID. NO. 21; ephrin B1 or B3/syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27; ephrin B1 or B3/ residues 1-299 of syntenin ; ephrin B1 or B3/PHIP PDZ2 of SEQ. ID. NO. 24, ephrin B1 or B3/PHIP PDZ3 of SEQ. ID. NO. 25.

5. A peptide derived from the PDZ binding domain of a B class ephrin.

6. A synthetic peptide of the formula I which interferes with the interaction of a B class ephrin and a PDZ domain containing protein:

$$X\text{-}X^1\text{-}X^2\text{-}K\text{-}V \qquad \text{I}$$

wherein X represents 0 to 70 amino acids, and each of $X^1$ and $X^2$ represent tyrosine or phosphotyrosine.

7. A peptide as claimed in claim 6 wherein X represents 2 to 20 amino acids.

8. A peptide as claimed in claim 7 wherein X represents NI, GNI, CPHYEKVSGDYGHPVYIVQ(E,D)(M,G)PPQSP(A,P)A (SEQ.ID. NO. 2), GDYGHPVYIVQ(E,D)(M,G)PPQSP(A,P)A(SEQ.ID. NO. 3), PPQSP(A,P)A (SEQ.ID. NO. 4), GPPQSPPNI (SEQ.ID. NO. ).

9. A peptide as claimed in claim 7 which is YYKV (SEQ ID. NO. 5), GPPQSPPNIpYYKV (SEQ ID. NO. 6), NIpYpYKV (SEQ ID. NO. 7), NIpYYKV (SEQ ID. NO. 8), NIYpYKV (SEQ ID. NO. 9), NIYYKV (SEQ ID. NO. 10), GNIYYKV (SEQ ID. NO. 28), GNIpYpYKV (SEQ ID. NO. 29), GNIpYYKV (SEQ ID. NO. 30 ), or GNIYpYKV (SEQ ID. NO. 31).

10. A complex comprising a peptide as claimed in claim 6, 7, 8, or 9 and a PDZ domain containing protein.

11. A complex as claimed in claim 10 wherein the PDZ domain containing protein is GRIP,

GRIP PDZ6 and PDZ 7 of SEQ.ID.NO. 22 and 23, FAP-1 PDZ of SEQ. ID. NO. 21, amino acids residues 1 to 299 of syntenin, syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27, PHIP PDZ2 of SEQ. ID. NO. 24; and PHIP PDZ3 of SEQ. ID. NO. 25.

12. A complex as claimed in claim 10 which is FAP-1 PDZ of SEQ. ID. NO. 21/NIpYYKV, FAP-1 PDZ of SEQ. ID. NO. 21/NIpYpYKV, syntenin/NIYYKV, syntenin/NIpYYKV, syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27/NIYYKV, syntenin PDZ1 and PDZ2 of SEQ. ID. NO. 26 and 27/ NIpYYKV, PHIP PDZ3 of SEQ. ID. NO. 25/GNIpYpYKV, or PHIP PDZ3 of SEQ. ID. NO. 25/GNIpYYKV.

13. A method of modulating the interaction of a B class ephrin and a PDZ domain containing protein comprising administering an effective amount of a complex as claimed in claim 1.

14. A method of modulating the interaction of a B class ephrin and a PDZ domain containing protein comprising administering an effective amount of a peptide as claimed in claim 6.

15. A method for identifying a substance that binds to a complex as claimed in claim 1 comprising: (a) reacting the complex with at least one substance which potentially can bind with the complex, under conditions which permit binding of the substance and complex; and (b) detecting binding, wherein detection of binding indicates the substance binds to the complex.

16. A method as claimed in claim 15 wherein binding is detected by assaying for substance-complex conjugates, or for activation of the B class ephrin B or PDZ domain containing protein

17. A method for evaluating a compound for its ability to modulate the interaction of a B class ephrin and a PDZ domain containing protein which comprises providing a complex as claimed in claim 1, 2 or 3, with a substance which binds to the complex, and a test compound under conditions which permit the formation of conjugates between the substance and complex, and removing and/or detecting conjugates.

18. A method for evaluating a compound for its ability to modulate the interaction of a B class ephrin and a PDZ domain containing protein which comprises (a) providing a B class ephrin and a PDZ domain containing protein, and a test compound, under conditions which permit binding of the B class ephrin and PDZ domain containing protein; and (b) detecting binding, wherein the detection of increased or decreased binding relative to binding in the absence of the test compound indicates that the test compound modulates the interaction of a B class ephrin and a PDZ domain containing protein.

19. A method of modulating the interaction of a B class ephrin and a PDZ domain containing protein comprising changing the terminal amino acid Val in a B class ephrin.

20. Use of a complex as claimed in claim 1 or a peptide as claimed in claim 6 in the preparation of a medicament to modulate the interaction of a B class ephrin and a PDZ domain containing protein.

21. Use of a complex as claimed in claim 1 or a peptide as claimed in claim 6 in the preparation of a medicament to modulate cellular processes of cells associated with B class ephrins or PDZ domain containing proteins.

22. A use as claimed in claim 21 wherein the cellular processes are axonogenesis, nerve cell interactions, and regeneration of nerve cells.

23. A composition comprising a complex as claimed in claim 1 or a peptide as claimed in claim 6, and a pharmaceutically acceptable carrier, excipient or diluent effective for administration to individuals suffering from disorders associated with a B class ephrin.

24. A method for modulating proliferation, growth, or differentiation of cells associated with B class ephrins or PDZ domain containing proteins comprising introducing into the cells a complex as claimed in claim 1 or a peptide as claimed in claim 6.

25. A method for treating proliferative or differentiative disorders associated with B class ephrins or PDZ domain containing proteins using a composition as claimed in claim 23.

26. An isolated protein comprising the amino acid sequence of SEQ. ID. NO.1.

27. A truncation, an analog, an allelic or species variation of a protein as claimed in claim 26, or a protein having substantial sequence identity with the protein as claimed in claim 26.

28. A fusion protein comprising an isolated protein as claimed in claim 26 conjugated to a protein.

29. Antibodies having specificity against an epitope of a protein as claimed in claim 26.

30. A method for identifying a substance which binds to a protein as claimed in claim 26 comprising reacting the protein with at least one substance which potentially can bind with the protein, under conditions which permit the binding of the substance and protein, and detecting binding, wherein the detection of binding indicates that the substance binds to the protein.

31. A method for evaluating a compound for its ability to modulate the biological activity of a protein as claimed in claim 26 comprising providing the protein, a substance which binds to the protein, and a test compound under conditions which permit binding of the substance and protein, and detecting binding, wherein the detection of increased or decreased binding relative to binding detected in the absence of the test compound indicates that the test compound modulates the activity of the protein.

# Figure 1

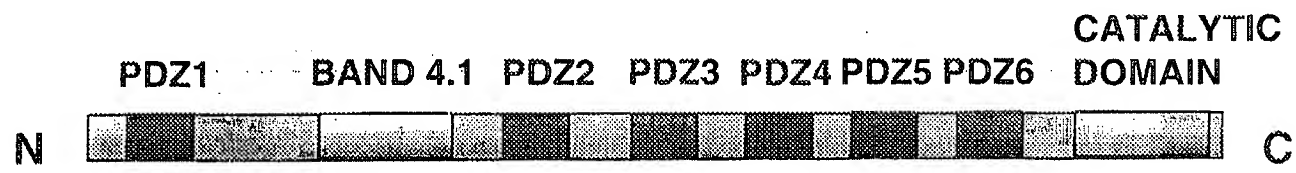
```
Ephrin B1   LRKRHRKHTQQRAAALSLS...TLASPKGGS.    292
Ephrin B2   YRRRHRKHSPQHTTTLSLS...TLATPKRSG.    279
Ephrin B3   RRRRAKPSESRHPGPGSFGRGGSLGLGGGGGM    283

Ephrin B1   GTAGTEPSDIIIPLR...TTENNYCPHYEKVS    321
Ephrin B2   NNNGSEPSDIIIPLR...TADSVFCPHYEKVS    308
Ephrin B3   GPREAEPGELGIALRGGGAADPPFCPHYEKVS    315
                                     *

Ephrin B1   GDYGHPVYIVQEMPPQSPANIYYKV—COOH      346
Ephrin B2   GDYGHPVYIVQEMPPQSPANIYYKV—COOH      333
Ephrin B3   GDYGHPVYIVQDGPPQSPPNIYYKV—COOH      340
              *    *             **
```

# Figure 2A

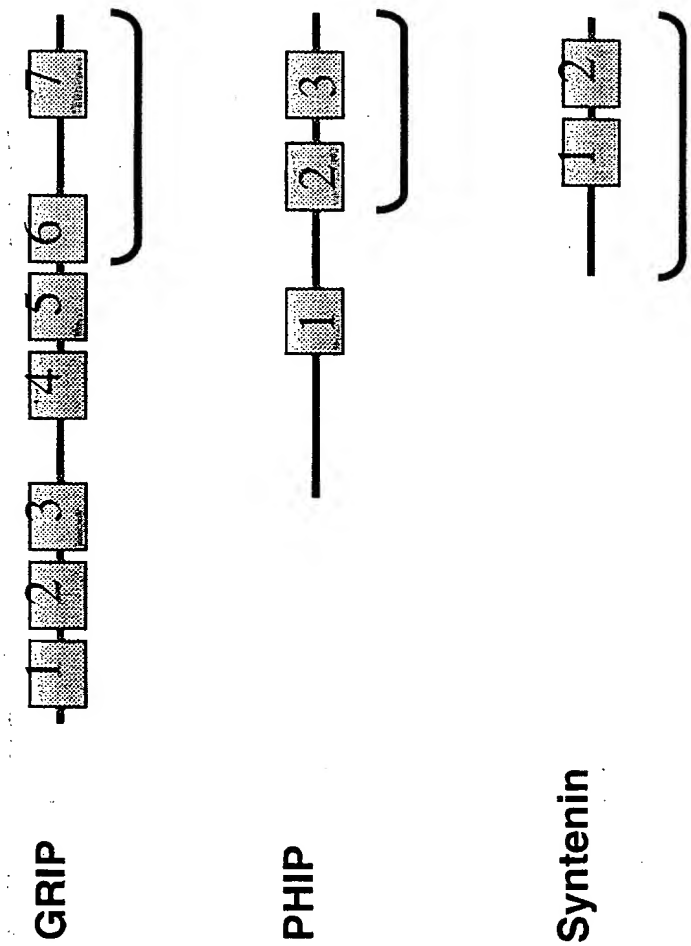
FAP-1




Binding specificity of FAP-1 PDZ5 as deduced through oriented peptide libraries:

| Amino acid position relative to C-terminus | -8 | -7 | -6 | -5 | -4 | -3 | -2 | -1 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| Amino acid residue | - | X | X | X | E | I | Y | Y | V |
| | | | | | | Y | | K | K |
| | | | | | | V | | | I |

# Figure 2B



# Figure 2C

```
FAP-1 PDZ5      LITLIKSEK..AS.LGFTVTK.G..N..QRIG
GRIP PDZ6       TVELKRYG...GP.LGITISG.TEEP...FDP
GRIP PDZ7       VTLYKDFGM..ED.FGFSVAD.G..L..LEKG
PHIP PDZ2       NIQLKK.GT..EG.LGFSITS.RDVTIGGSAP
PHIP PDZ3       EVPLNDSGS..AG.LGVSVKGNRSKENHADLG
SYNTENIN PDZ1   REVILCKDQD..GKIGLRLKS.ID......NG
SYNTENIN PDZ2   RTITMHKDST..GHVGFIFK...NG......K

FAP-1 PDZ5      CYVHDV.IQDPAKSDGRLKPGDRLIKVNDTDV
GRIP PDZ6       IIISSLTKGGLAERTGAIHIGDRILAINSSSL
GRIP PDZ7       VYVKNIRPAGPGD.VGGLKPYDRLLQVNHVRT
PHIP PDZ2       IYVKNILPRGAAIQDGRLKAGDRLIEVNGVDL
PHIP PDZ3       IFVKSIINGGAASKDGRLRVNDQLIAVNGESL
SYNTENIN PDZ1   IFVQLVQANSPASLVG.LRFGDQVLQINGENC
SYNTENIN PDZ2   IT.SIVKDS.SAARNG.LLTEHNICEINGQNV

FAP-1 PDZ5      TNM..THTDAVNLLRAASK..TVRLVIGR
GRIP PDZ6       KGK.PLSEAIHLLQMAGE..TVTLKIKK
GRIP PDZ7       RDF.DCCLVVPLIAESGN..KLDLVISR
PHIP PDZ2       AGK.SQEEVVSLLRSTKMEGTVSLLVFR
PHIP PDZ3       LGKANQEAMETLRRSMSTEGNKRGMIQL
SYNTENIN PDZ1   AGW.SSDKAHKVLKQA.FGEKITMTIRD
SYNTENIN PDZ2   IGL.KDSQIADILSTSGTVVTITIMPAF
```

# Figure 2D

```
PHIP   M.................................................
PAR-3  MSASSTSSSSTSCPEGGEPSGSCKSSDEGESTLKKRMQQYGIASGYANS

PHIP   ...................KVTVCFGRTRVVVPCGDGRMKVFSLIQQAV
PAR-3  SISTLDRSQYQSLPLNGTRRVTVQFGRMKIVVPWKESDQTVGQLADAAL

PHIP   TRYRKAVAKDPNYWIQVHRLE.HGDGGILDLDDILCDVAD.DKDRLVAV
PAR-3  LRYKKARGMANEDRIHVHRLECASDGGILDMDDVLEEVFDLNYDQILAI

PHIP   FDEQD....P.........HH............GGDGTS.ASSTGT..
PAR-3  TDEANGGSTTPTYSQIQKQQHHYAQPLPYARKFDGGPSTPIASAFGSVT

PHIP   ........QSPEIFGSELGTNNVSAFQPYQATSEIEVTPSVLRAN....
PAR-3  VNHQAHRAASPYNVG..FARSNSRDFAP.QPTHSKERRDSVVEVSSFDQ

PHIP   MPLHVRRSSDPALTGLSTSVSDNNFSSEEPSRKNPTRWSTT.....AG
PAR-3  IPQSGLRVSTPKPSRQSEDVIDGK.PMNQPILRSSLRTEASGSRTEEAT

PHIP   FLKQNTAG.SPKTC......DRKKDENYRSLPRDPSSWSNQEQRDNARS
PAR-3  PVKQSRVTLSPEVEKKLAEQDERKSERRKHYDKNPG....RFARGSDRK

PHIP   S..LSASHPMVDRWLEKQEQDEEGTEEDSSRVEPVGHADTGLENMPNFS
PAR-3  SRITDALLDARDR.IADQLESQNPAEETKSQMIRV.KIDQG..PMPGTS

PHIP   LDDMVKLVQVPNDGGPLGIHV.........VPFSARGGRTLGLLVKRLE
PAR-3  LVTFPPIPEKSENEKQLGIEVNAVFDESSELPGTSEPTKLSSVQIMKIE

PHIP   KGGKAEQENLFHENDCIVRINDGDLRNRRFEQAQHMFRQAMRARVIWFH
PAR-3  DGGRIAKDGRIRVGDCIVAI.DG.......KPVDQM..SIIRVR.....

PHIP   VVPAANKEQYEQLSQREKNNYSPGRFSPDSHCVANRSVANNAPQALPRA
PAR-3  ....ASISDLAAVTSR...........PVT.LIINRSLESFLEQE....

PHIP   PRLSQPPEQLDAHPRLPHSAHASTKPPAAPALAPPSVLSTNVGSVYNTK
PAR-3  ....................SSAKPIQSALQQA..........NTQ

PHIP   KVGKRLNIQLKKGTEGLGFSITSRDVTIGGSAPIYVKNILPRGAAIQDG
PAR-3  YIGHTTVVELIKSSNGFGFTVTGRE.TAKGERLFYIGTVKPYGVAL..G

PHIP   RLKAGDRLIEVNGVDLAGKSQEEVVSLLRSTKMEGTVSLLVFR......
PAR-3  HLKSGDRLLEINGTPTGQWTQSEIVEKLKETMVGEKIKFLVSRVSQSAI

PHIP   ...........QEEAFHPREMNAEPSQMQTPK.ETKAEDEDV.VLTPDG
PAR-3  MSTSASSENKENEETLKVVEEEKIPQKLPLPALMTPPVPKDTPALSPSG

PHIP   TREFLTFEVP.LNDSGSAGLGVSVKGNRSKENHA...DLGILFVKSIING
PAR-3  ASRF.EIVIPFINGSSSAGLGVSLKARVSKKSNGSKVDCGIFIKNVMHG
```

# Figure 2D (cont'd)

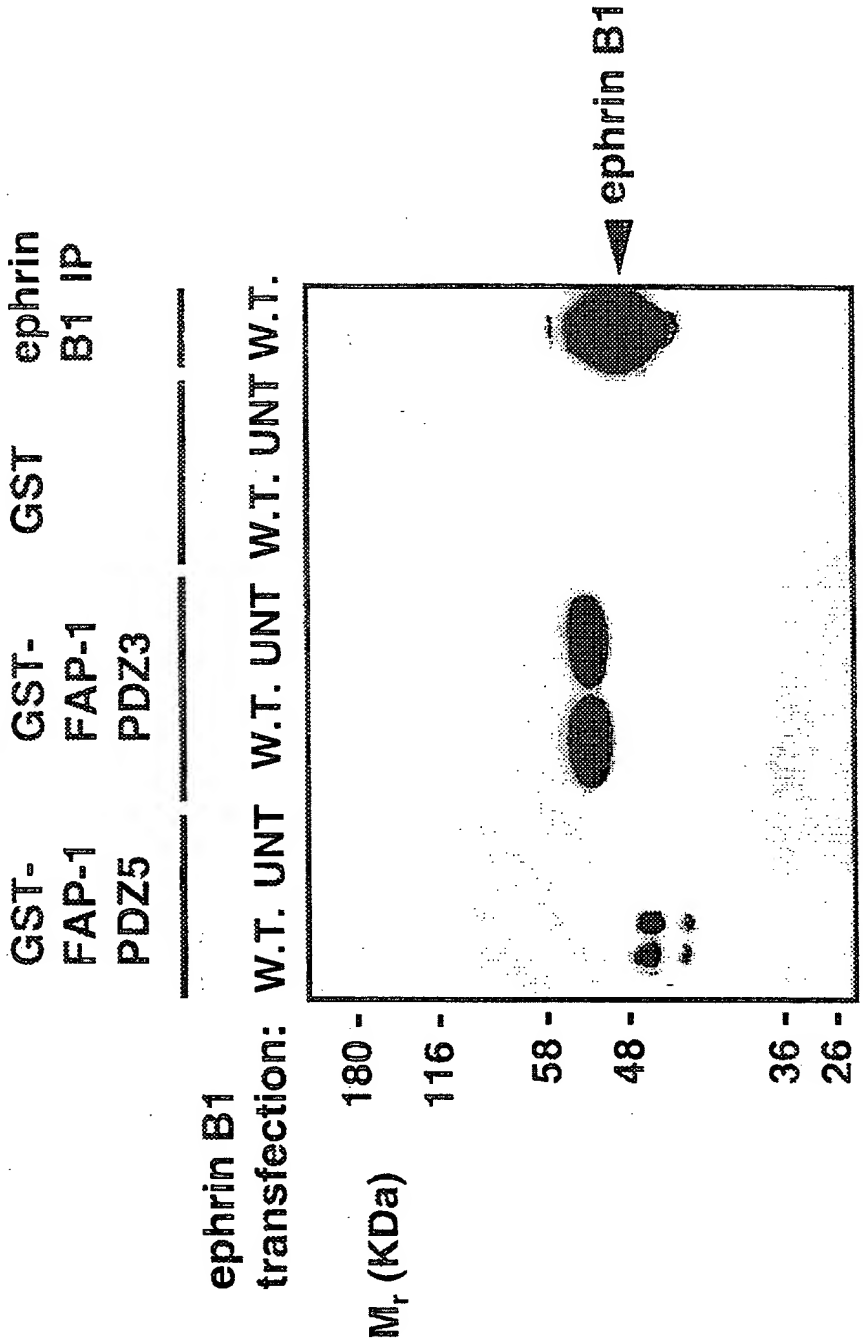
```
PHIP  GAASKDGRLRVNDQLIAVNGESLLGKANQEAMETLRRSMSTEGNKRGMI
PAR-3 GAAFKEGGLRVDDRIVGVEDIDLEPLDNREAQAALAKKLK....EVGMI

PHIP  QLIVARRISRCNELRSPGSPAAPELPIETELDDRERRISHSLYSGIEGL
PAR-3 SSNVRLTISRYNEC.NPGQ.......ISRDLS....RIT......VDAS

PHIP  DESPTRNAALSRIMGESGT
PAR-3 SPSPS.....SRMSSHTAPDSLLPSPATRGTSSSGADSSHSRQSSASSA

PAR-3 VPAVPARLTERDSIVSDGTSRNDESELPDSADPFNREGLGRKSLSEKRG
PAR-3 MGAAADPQHIKLFQDIKHQRQNSAPTSSTQKRSKSQPRSSSQRNYRSPM
PAR-3 KLVDLPTTAAASASTNSQNLDDSDMLNRRSQSMESINRPVESILRGTGQ
PAR-3 IPTGSSSKVQFMQAASPDQHPFPPGAALLRLKNEESRSRDKSRRKSMGN
PAR-3 PFSAMRNFFGFGSKSRDASPEKTPTESVQLRSVERPKSIIDERNNGSSE
PAR-3 RAPPPLPPHQSQRRGSGGNVFVDYGEPYGLIPQYPHNTTSGYESYADSE
PAR-3 LYDRYAAHRYHPRGGPIIDEDEYIYRQQSTSGNSPINTSSYVNYGLPAS
PAR-3 NAYHVGSRIPPQTSSGSISKTSGAMRRVYPAEYYDEDVAYHQQIPQQST
PAR-3 RYQQGSGSGRGNADYHHMFNSWFAYTGGGAVGAAPVIKSSYGSSPVRIA
PAR-3 AASAIERGESFVVEPVSGSSASATDRRGRSTSSGAVASGSSSTGFQYAA
PAR-3 KEKYADARSGKFNGGSTRLFIPRHGGGLSAAAFATNFGGEAYETRGGGA
PAR-3 GGSPSQYRRRDQGPPHRFPQY
```

Figure 3A


GST-
FAP-1
PDZ5
GST-
FAP-1
PDZ3
GST
ephrin
B1 IP
ephrin B1
transfection:
W.T. UNT W.T. UNT W.T. UNT W.T.
Mr (KDa)
180 -
116 -
58 -
48 -
36 -
26 -
ephrin B1

# Figure 3B

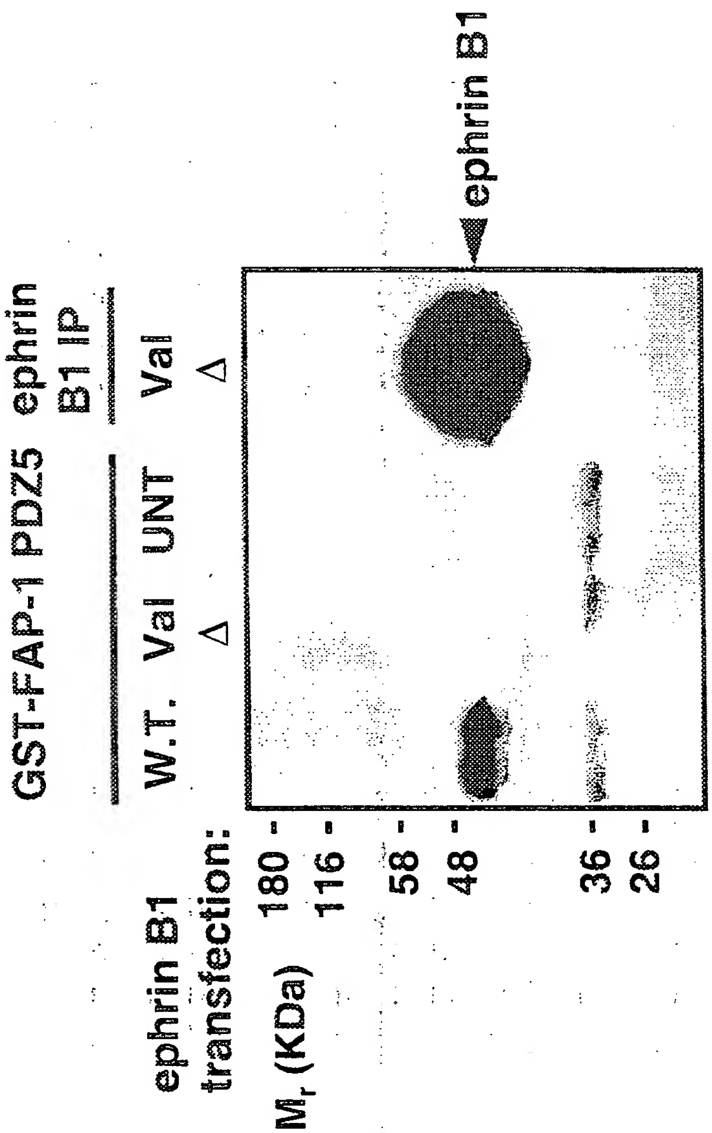
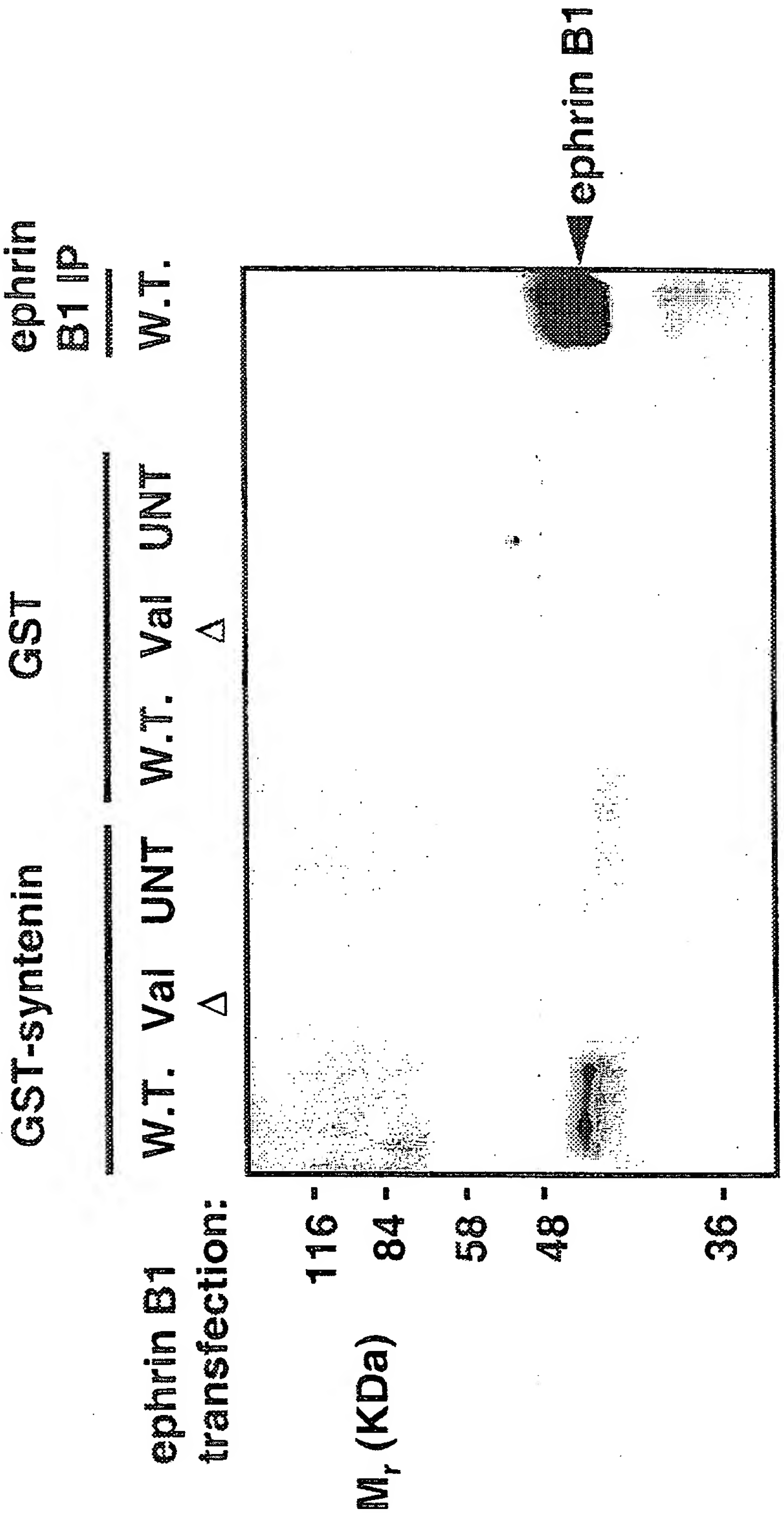

GST-FAP-1 PDZ5
ephrin B1 IP
ephrin B1 transfection:
W.T.
Val Δ
UNT
Val Δ
Mr (KDa)
180
116
58
48
36
26
ephrin B1

Figure 3C


GST-syntenin
GST
ephrin B1 IP
ephrin B1 transfection:
W.T.
Val Δ
UNT
W.T.
Val Δ
UNT
W.T.
ephrin B1
$M_r$ (KDa)
116
84
58
48
36

# Figure 3D

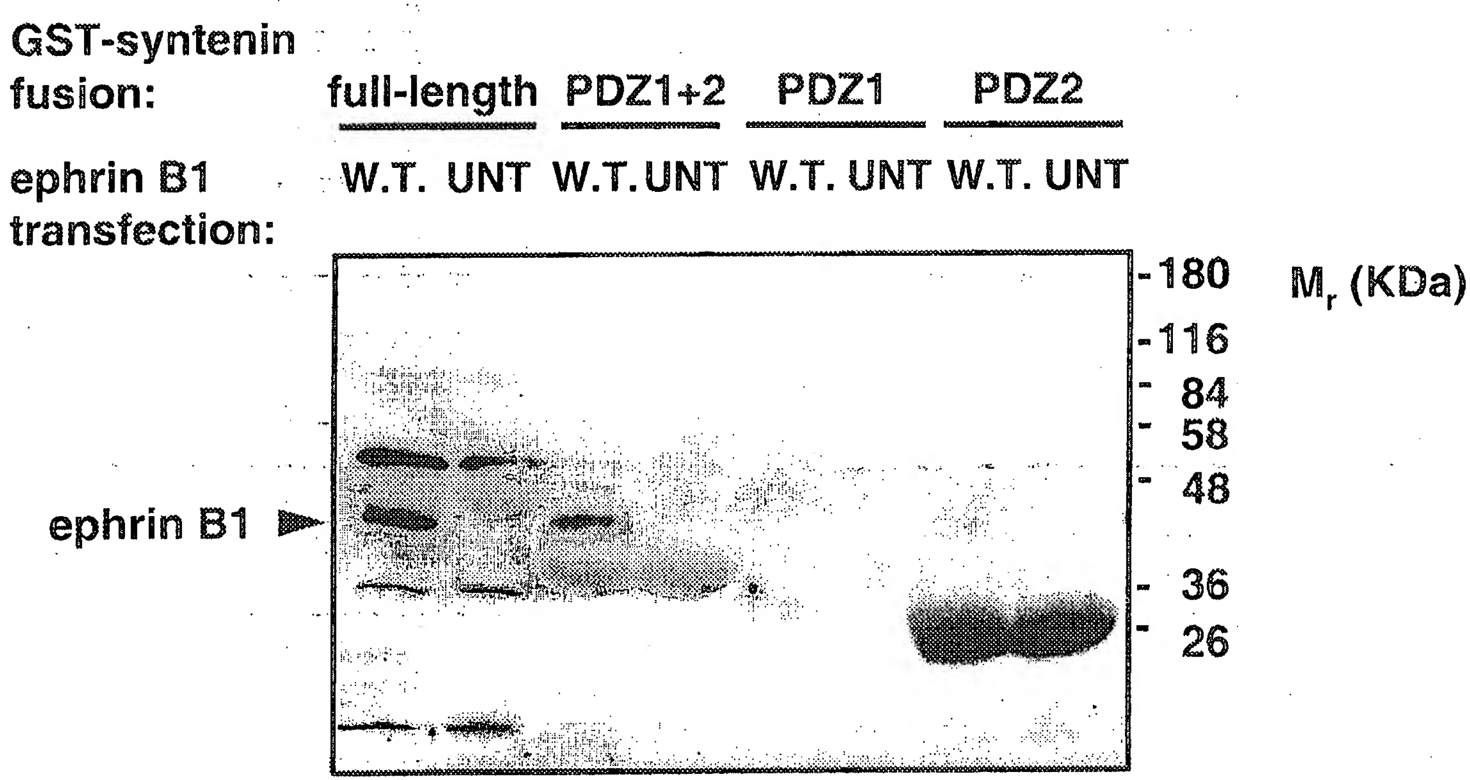
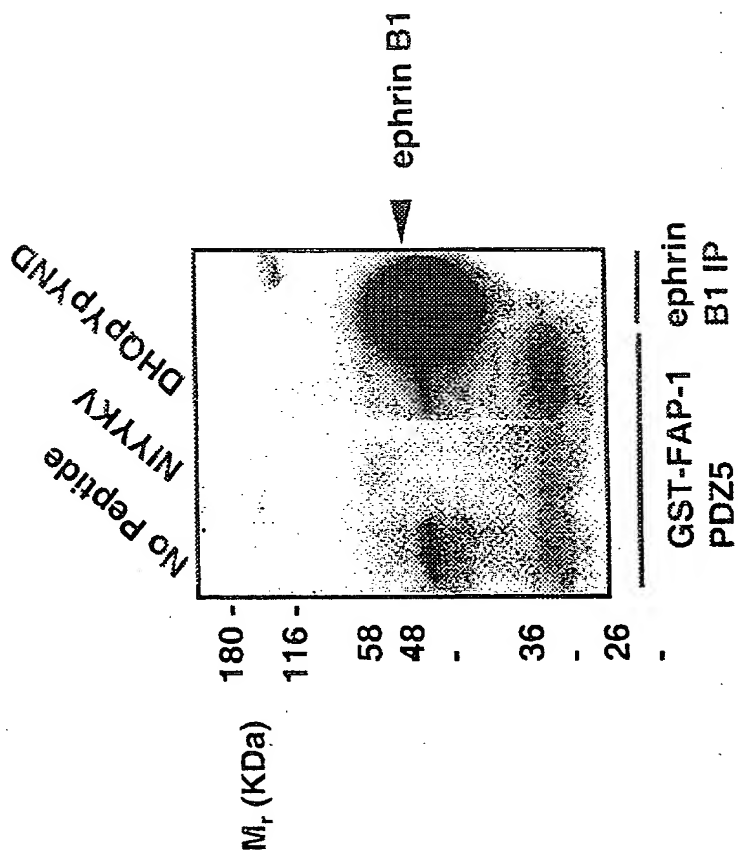

GST-syntenin fusion:
full-length
PDZ1+2
PDZ1
PDZ2
ephrin B1 transfection:
W.T. UNT
W.T. UNT
W.T. UNT
W.T. UNT
ephrin B1
180
116
84
58
48
36
26
$M_r$ (KDa)

Figure 4A



ephrin B1
DHQpYpYND
NIYYKV
No Peptide
180 -
116 -
58
48
36
26
$M_r$ (KDa)
GST-FAP-1 PDZ5
ephrin B1 IP

Figure 4B

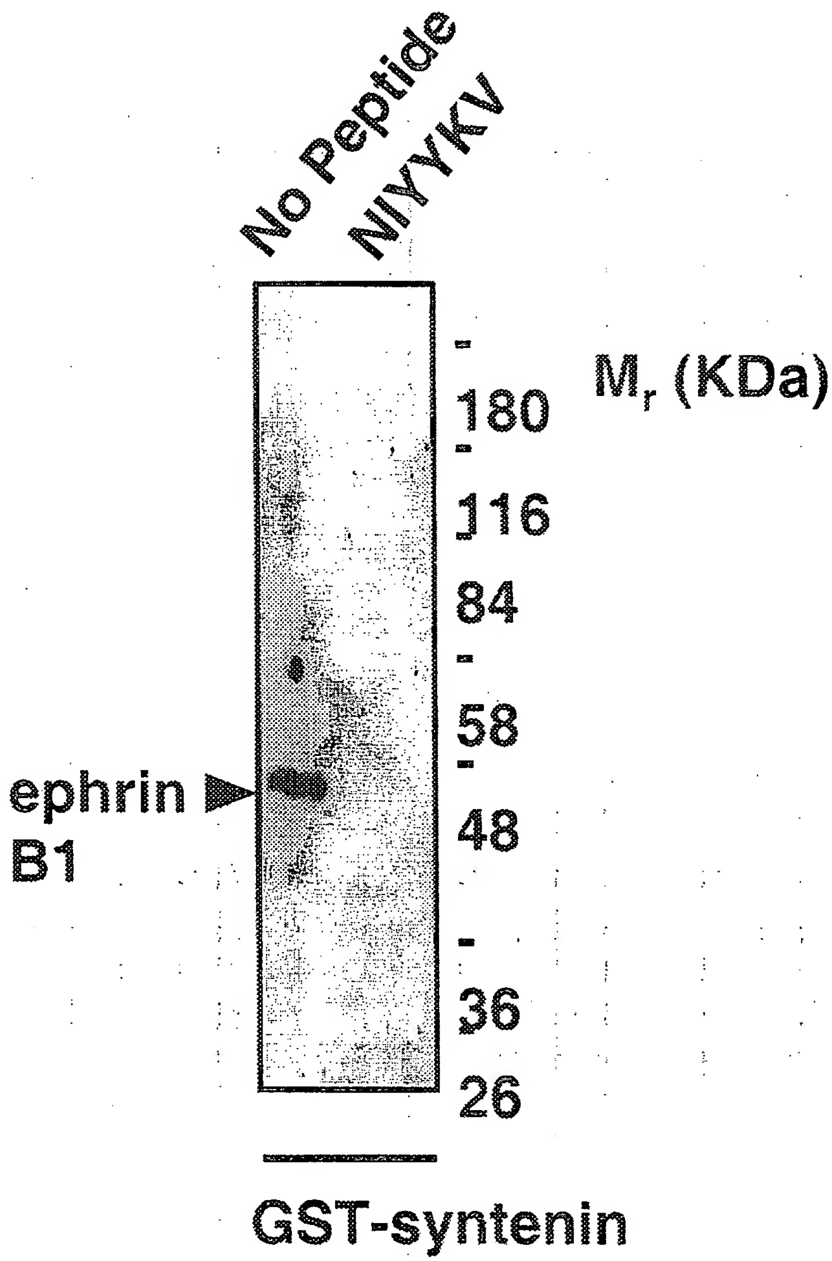

No Peptide
NIYYKV
$M_r$ (KDa)
180
116
84
58
48
36
26
ephrin B1
GST-syntenin

Figure 5A

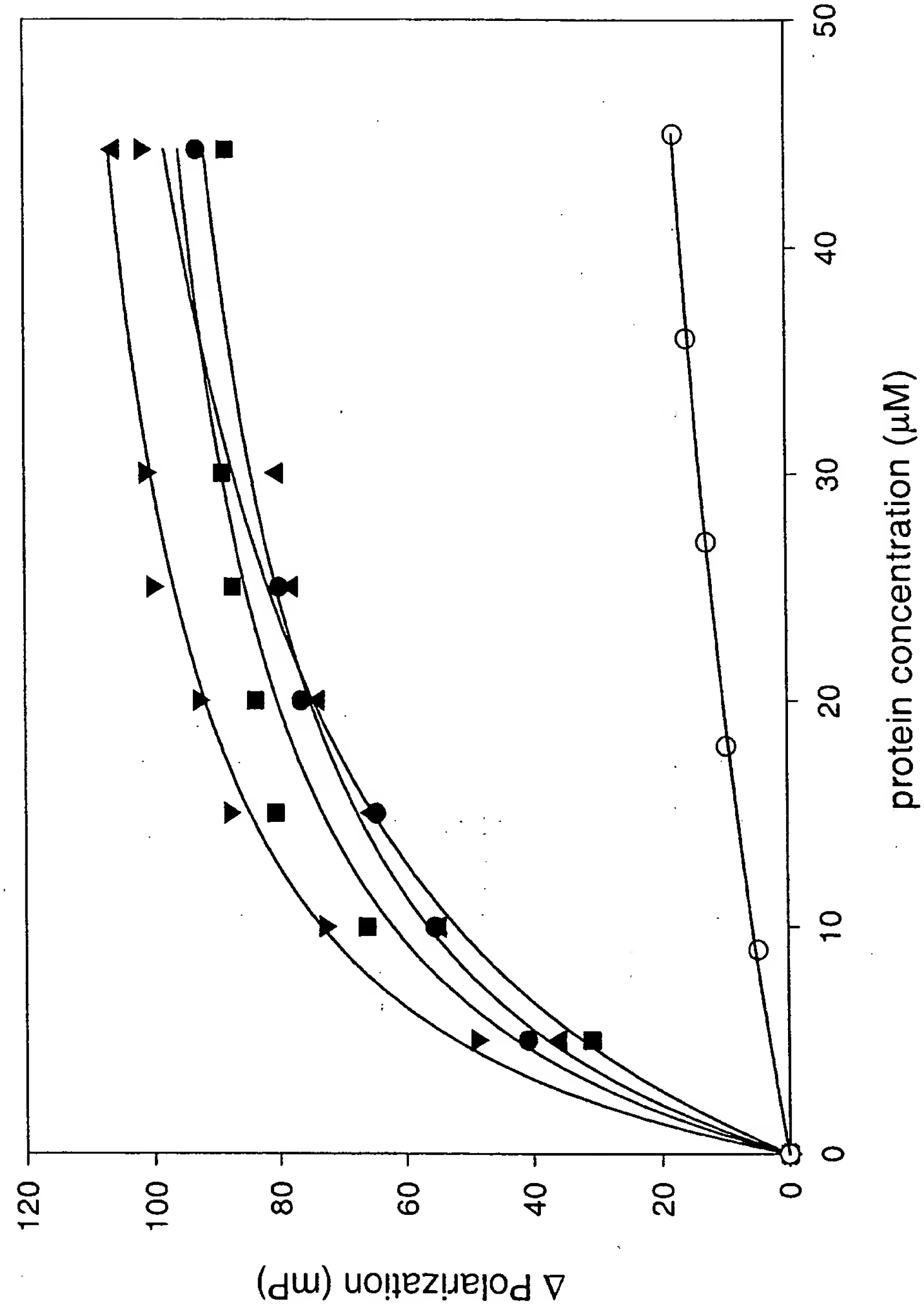

Δ Polarization (mP)
0
20
40
60
80
100
120
protein concentration (μM)
0
10
20
30
40
50

Figure 5B

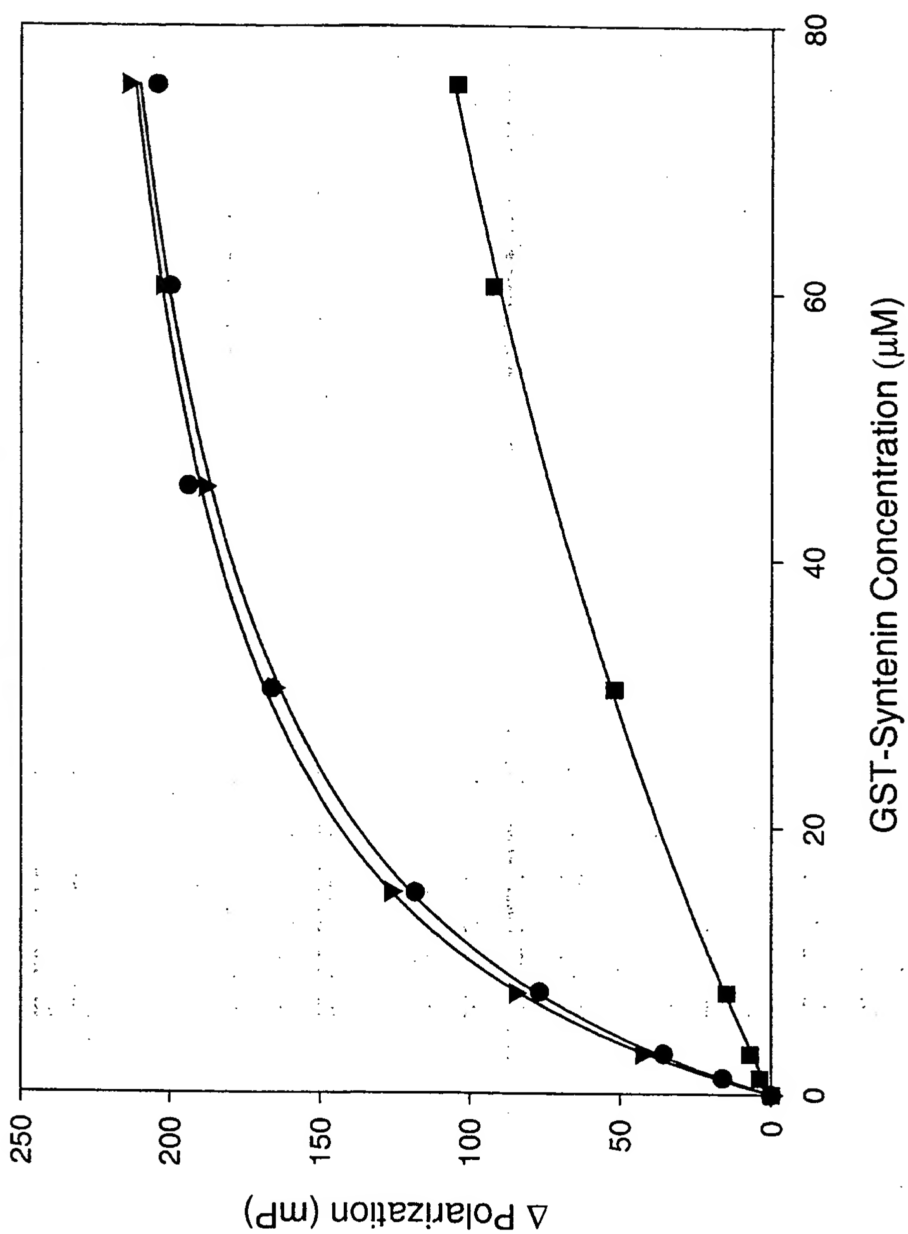

Δ Polarization (mP)
0
50
100
150
200
250
0
20
40
60
80
GST-Syntenin Concentration (μM)

Figure 6

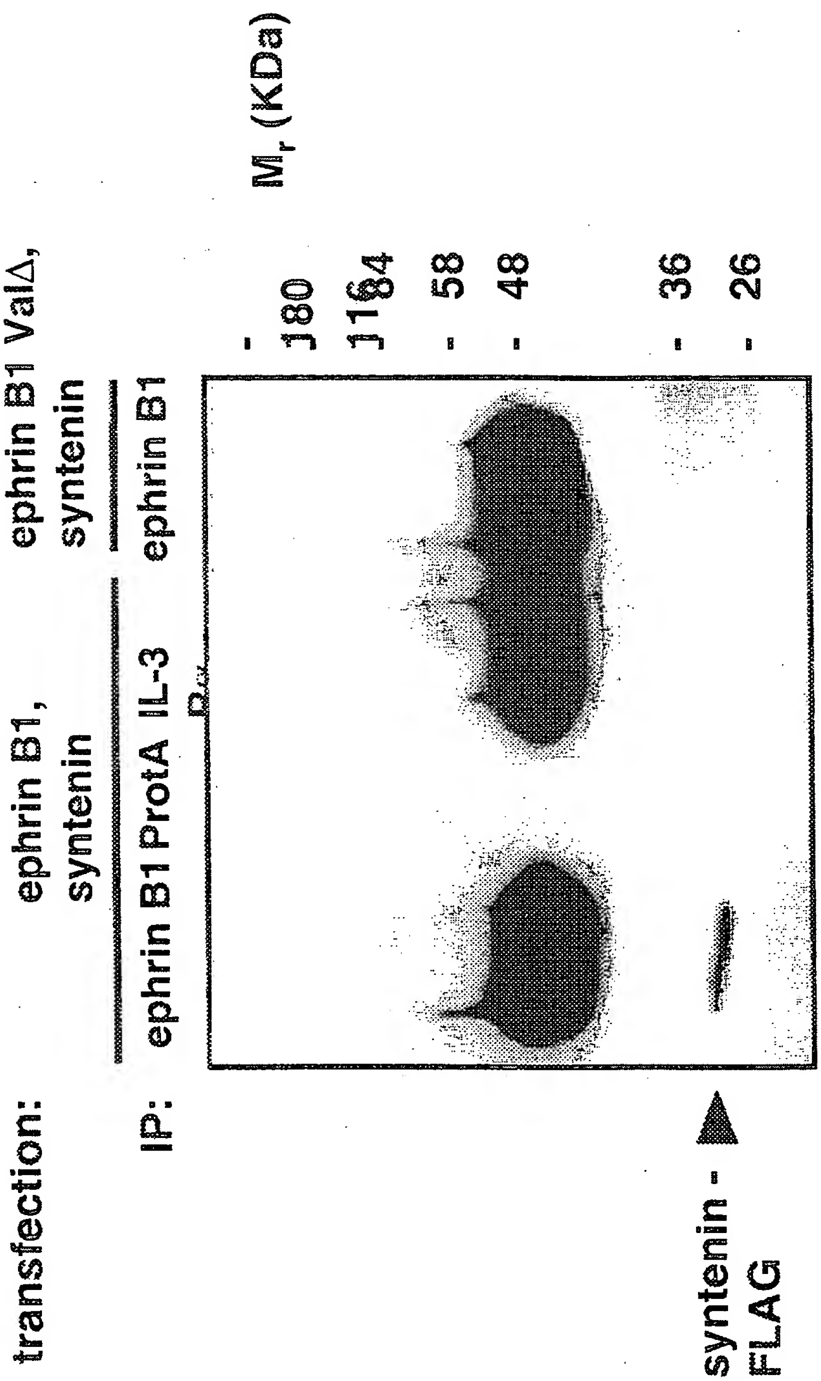

transfection:
ephrin B1, syntenin
ephrin B1 ValΔ, syntenin
IP:
ephrin B1
ProtA
IL-3
ephrin B1
ephrin B1
M_r (KDa)
180
116
84
58
48
36
26
syntenin - FLAG

**Figure 7**

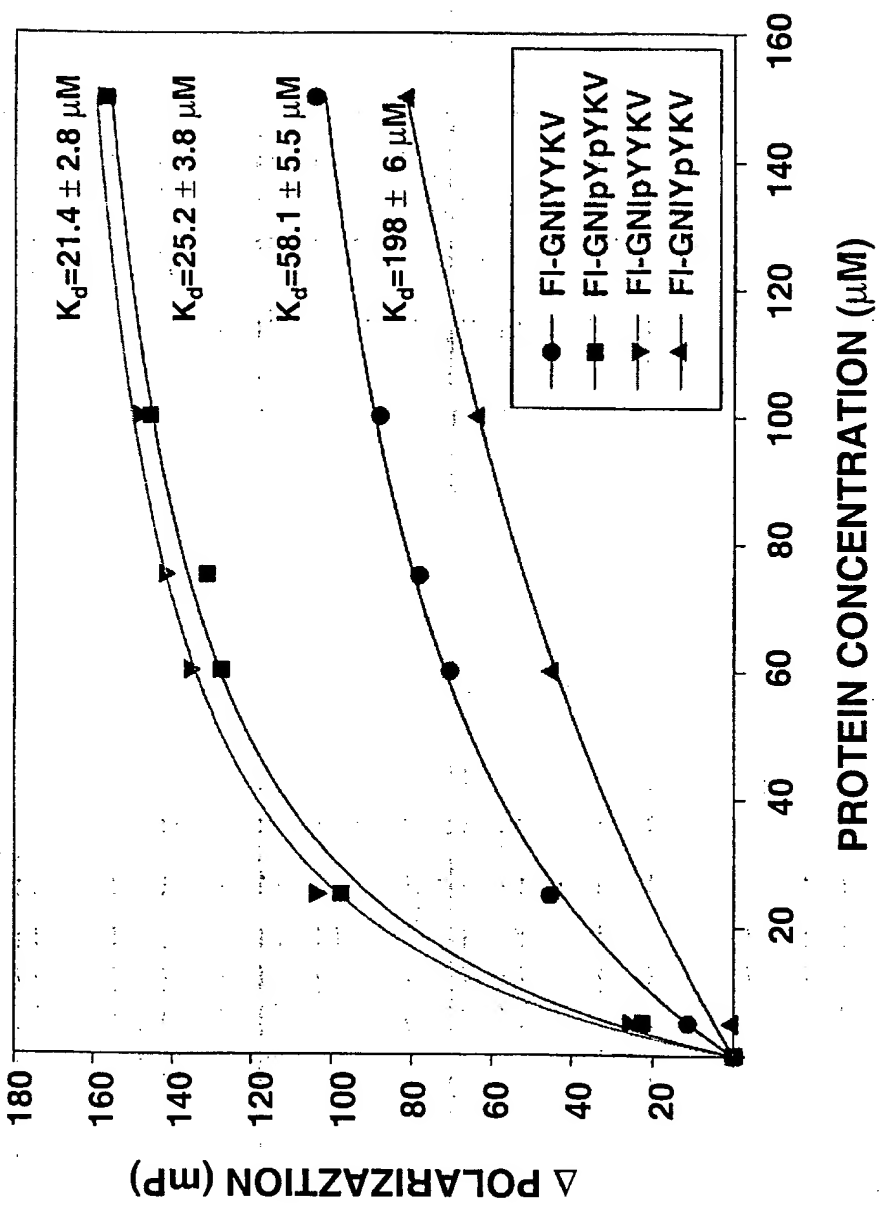
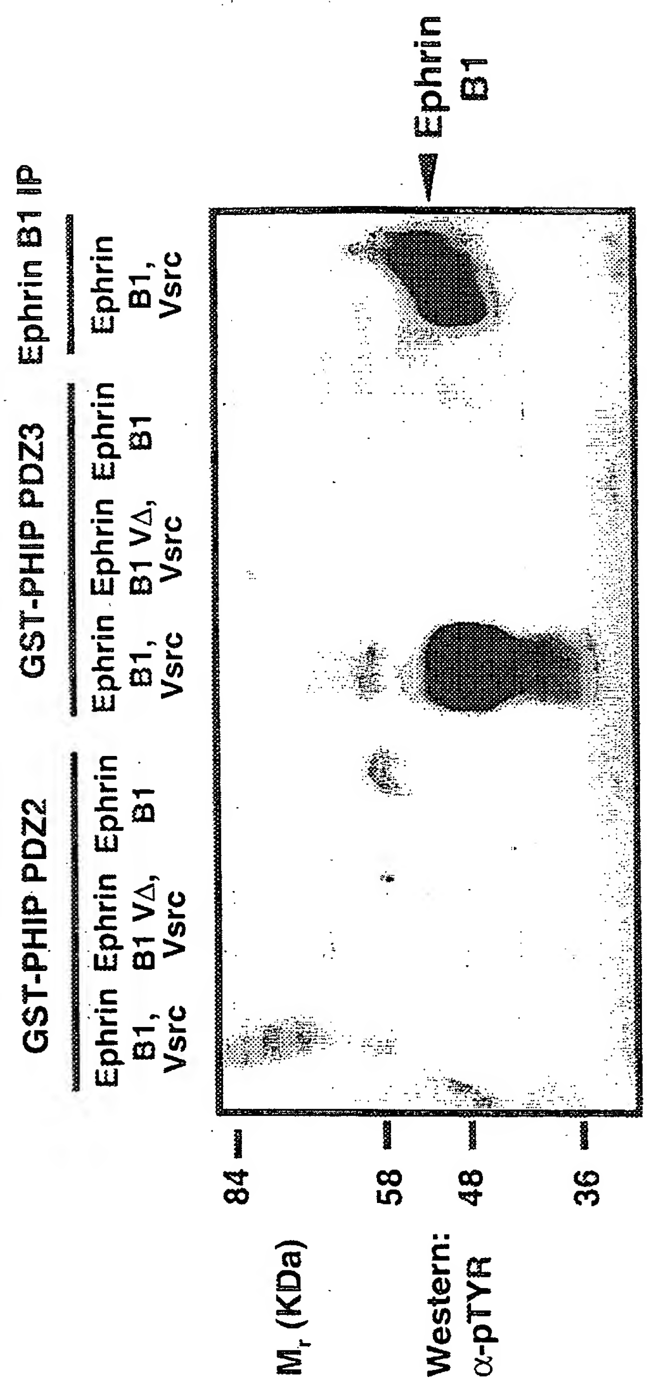

$K_d$=21.4 ± 2.8 μM
$K_d$=25.2 ± 3.8 μM
$K_d$=58.1 ± 5.5 μM
$K_d$=198 ± 6 μM
FI-GNIYYKV
FI-GNIpYpYKV
FI-GNIpYYKV
FI-GNIYpYKV
Δ POLARIZAZTION (mP)
PROTEIN CONCENTRATION (μM)
180
160
140
120
100
80
60
40
20
20
40
60
80
100
120
140
160

Figure 8


GST-PHIP PDZ2
GST-PHIP PDZ3
Ephrin B1 IP
Ephrin B1, Vsrc
Ephrin B1 V∆, Vsrc
Ephrin B1
Ephrin B1, Vsrc
Ephrin B1 V∆, Vsrc
Ephrin B1
Ephrin B1, Vsrc
Ephrin B1
84
58
48
36
Mr (KDa)
Western: α-pTYR

## Sequence Listing

SEQ. ID. NO. 1

MKVTVCFGR TRVVVPCGDG RMKVFSLIQQ AVTRYRKAVA KDPNYWIQVH
RLEHGDGGIL DLDDILCDVA DDKDRLVAVF DEQDPHHGGD GTSASSTGTQ
SPEIFGSELG TNNVSAFQPY QATSEIEVTP SVLRANMPLH VRRSSDPALT
GLSTSVSDNN FSSEEPSRKN PTRWSTTAGF LKQNTAGSPK TCDRKKDENY
RSLPRDPSSW SNQFQRDNAR SSLSASHPMV DRWLEKQEQD EEGTEEDSSR
VEPVGHADTG LENMPNFSLD DMVKLVQVPN DGGPLGIHVV PFSARGGRTL
GLLVKRLEKG GKAEQENLFH ENDCIVRIND GDLRNRRFEQ AQHMFRQAMR
ARVIWFHVVP AANKEQYEQL SQREKNNYSP GRFSPDSHCV ANRSVANNAP
GALPRAPRLS QPPEQLDAHP RLPHSAHAST KPPAAPALAP PSVLSTNVGS
VYNTKKVGKR LNIQLKKGTE GLGFSITSRD VTIGGSAPIY VKNILPRGAA
IQDGRLKAGD RLIEVNGVDL AGKSQEEVVS LLRSTKMEGT VSLLVFRQEE
AFHPREMNAE PSQMQTPKET KAEDEDVVLT PDGTREFLTF EVPLNDSGSA
GLGVSVKGNR SKENHADLGI FVKSIINGGA ASKDGRLRVN DQLIAVNGES
ILGKANQEAM ETLRRSMSTE GNKRGMIQLI VARRISRCNE LRSPGSPAAP
ELPIETELDD RERRISHSLY SGIEGLDESP TRNAALSRIM GESGT

SEQ.ID. NO. 2

CPHYEKVSGDYGHPVYIVQ(E,D)(M,G)PPQSP(A,P)A

SEQ.ID. NO. 3

GDYGHPVYIVQ(E,D)(M,G)PPQSP(A,P)A

SEQ.ID. NO.4

PPQSP(A,P)A

SEQ ID. NO. 5

YYKV

SEQ ID. NO. 6

GPPQSPPNIpYYKV

SEQ ID. NO. 7

NIpYpYKV

SEQ ID. NO.8

NIpYYKV

SEQ ID. NO. 9

NIYpYKV

SEQ ID. NO. 10

NIYYKV

SEQ. ID. NO. 11

DHQpYpYND

SEQ. ID. NO. 12

PYpYKV

SEQ. ID. NO. 13

GNIYYKV

SEQ. ID. NO. 14

AYYY

SEQ. ID. NO. 15

LRKHHRKHTQQRAAALSLSTLASPKGGSGTA GTEPSDIIIPLRTTENNYCPHYEKVS
GDYGHPVYIVQEMPPQSPANIYYKV

SEQ. ID. NO. 16

YRRHHRKHSPQHTTTLSLSTLATPKRGGNNN GSEPSDVIIPLRTADSVFCPHYEKVS
GDYGHPVYIVQEMPPQSPANIYYKV

SEQ. ID. NO. 17

RRRHAKPSESRHPGPGSFGRGGSLGLGGGGGM
GPREAEPGELGIALRGGGTADPPFCPHYEKVS
GDYGHPVYIVQDGPPQSPPNIYYKV

SEQ. ID. NO. 18

XXXEIYYV

SEQ. ID. NO. 19

XXXEYVYKK

SEQ. ID. NO. 20

XXXEVYYI

SEQ. ID. NO. 21

FAP-1 PDZ5

LITLKSEKASLGFTVTKGNQRIG CYVHDVIQDPAKSDGRLKPGDRLIKVNDTDV
TNMTHTDAVNLLRAASKTVRLVIGR

SEQ. ID. NO. 22

GRIP PDZ6

TVELKRYGGPLGITISGTEEPFDP IISSLTKGGLAERTGAIHIGDRILAINSSSL
KGKPLSEAIHLLQMAGETVTLKIKK

SEQ. ID. NO. 23

GRIP PDZ7

VTLYKDFGMEDFGFSVADGLLEKG VYVKNIRPAGPGDVGGLKPYDRLLQVNHVRT
RDFDCCLVVPLIAESGNKLDLVISR

SEQ. ID. NO. 24

PHIP PDZ2

NIQLKKGTEGLGFSITSRDVTIGGSAP IYVKNILPRGAAIQDGRLKAGDRLIEVNGVDL
AGKSQEEVVSLLRSTKMEGTVSLLVFR

SEQ. ID. NO. 25

PHIP PDZ3

EVPINDSGSAGLGVSVKGNRSKENHADLG IFVKSIINGGAASKDGRLRVNDQLIAVNGESL
LGKANQEAMETLRRSMSTEGNKRGMIQL

SEQ. ID. NO. 26

SYNTENIN PDZ1

REVILCKDQDGKIGLRLKSIDNG IFVQLVQANSPASLVGLRFGDQVLQINGENC
AGWSSDKAHKVLKQAFGEKITMTIRD

SEQ. ID. NO. 27

SYNTENIN PDZ2

RTITMHKDSTGHVGFIFKNGK ITSIVKDSSAARNGLLTEHNICEINGQNV
IGLKDSQIADILSTSGTVVTITIMPAF

SEQ. ID. NO. 28

GNIYYKV

SEQ. ID. NO. 29

GNIpYpYKV

SEQ ID. NO.30

GNIpYYKV

SEQ ID. NO. 31

GNIYpYKV

SEQ.ID. NO. 32

GFPQSPPNI

SEQ. ID. NO. 33

PHIP na

1 TGGTGGTGCT GGTACCGGAT CGAATTCAAG CCGACTGAGC GCGAGCGCCA

51 CACGGCCCCG GGCCGCCGCC GAGCGCGCCA AGACGCCGAG ACGCCGAACA

101 GGTGGCCGGA GGCTGCAGGC GCCCGGGCGG GGACAGGCAA GGCCAGGCGA

151 AGGCGGCCGG GCTGGACATG GTAGCCGGGC AGCTCTGTGC GGCCGCCTGC

201 TCGCGCCTCT AGCCGCCGGC GCGCCGGCGC AGCGGCCCCA CCGCGCCCTG

251 CGTACAGTCT CCCGGCCCAG CGCCGCTCCG GCCACGGACA GCGAGGGACG

301 GCGGCATGAA AGTGACCGTG TGCTTCGGGA GGACCCGGGT GGTCGTGCCG

351 TGCGGAGATG GCCGCATGAA AGTTTTCAGC CTCATCCAGC AGGCGGTGAC

401 CCGCTACCGG AAGGCCGTGG CCAAGGATCC AAACTACTGG ATACAGGTGC

451 ATCGCTTGGA GCATGGAGAT GGAGGGATTC TAGACCTGGA TGACATCCTC

501 TGTGACGTTG CTGATGACAA AGACAGACTG GTAGCAGTAT TTGATGAACA

551 GGATCCCCAC CATGGAGGAG ATGGTACCAG CGCCAGCTCC ACGGGAACCC

601 AGAGTCCAGA GATATTCGGC AGTGAGCTGG GCACCAACAA TGTTTCTGCT

651 TTTCAGCCTT ATCAAGCCAC AAGTGAAATT GAGGTCACGC CTTCAGTTCT

701 TCGGGCAAAT ATGCCTCTTC ATGTCCGCCG GAGCAGCGAC CCAGCTTTAA

751 CTGGCCTTTC CACTTCTGTC AGTGATAACA ACTTTTCCTC AGAGGAGCCC

801 TCCAGGAAAA ACCCCACCCG CTGGTCCACG ACAGCTGGCT TTCTCAAGCA

851 GAACACCGCT GGAAGTCCCA AAACCTGCGA CAGGAAGAAA GATGAAAACT

901 ACAGAAGCCT TCCACGGGAT CCCAGTAGCT GGTCCAACCA GTTCCAGCGA

951 GACAACGCCC GCTCCTCCCT GAGCGCCAGC CACCCAATGG TAGACCGGTG

1001 GCTGGAGAAG CAAGAACAGG ATGAGGAAGG CACAGAAGAA GACAGCAGCC

1051 GAGTGGAGCC GGTTGGACAT GCTGATACCG GATTGGAGAA CATGCCCAAC

1101 TTTTCCCTCG ATGATATGGT AAAGCTCGTA CAAGTCCCCA ACGATGGAGG

1151 GCCCCTGGGA ATCCATGTAG TGCCTTTCAG TGCTCGAGGC GGCAGAACAT

1201 TGGGGTTGTT AGTGAAGCGG TTGGAGAAAG GCGGTAAGGC TGAGCAAGAA

1251 AACCTTTTCC ATGAGAATGA CTGCATTGTG AGGATTAACG ATGGAGATCT

1301 TCGAAACAGA AGATTTGAGC AAGCACAACA TATGTTCCGC CAAGCTATGC

1351 GTGCGCGTGT CATTTGGTTC CATGTGGTCC CTGCAGCAAA CAAGGAGCAA

1401 TATGAACAAC TGTCCCAACG CGAGAAGAAC AACTACTCCC CAGGCCGCTT

1451 CAGCCCTGAC AGCCACTGTG TGGCCAACAG GAGTGTGGCC AACAATGCCC

1501 CTCAAGCATT GCCCAGAGCA CCCAGACTGA GTCAGCCACC CGAGCAGCTG

1551 GATGCTCACC CCCGACTACC TCATAGTGCT CACGCCTCAA CCAAACCACC

1601 CGCAGCCCCG GCCTTGGCTC CACCCAGTGT GCTTAGTACC AACGTAGGCA

1651 GTGTGTACAA CACGAAGAAA GTAGGCAAGA GGCTCAACAT CCAGCTTAAG

1701 AAAGGTACAG AAGGACTGGG ATTCAGCATC ACCTCCCGGG ACGTCACCAT

1751 AGGTGGCTCA GCTCCCATTT ATGTCAAGAA TATCCTTCCT CGAGGGGCTG

1801 CCATTCAGGA TGGCAGACTC AAGGCAGGAG ACCGGCTAAT AGAGGTCAAT

1851 GGAGTAGATT TAGCAGGCAA ATCCCAGGAG GAAGTTGTTT CCCTGTTGAG

1901 AAGCACCAAG ATGGAGGGGA CTGTGAGCCT TCTGGTCTTT CGTCAGGAAG

1951 AGGCTTTCCA CCCAAGGGAA ATGAATGCTG AACCAAGCCA GATGCAGACT

2001 CCAAAAGAAA CGAAAGCTGA AGATGAGGAC GTTGTTCTCA CACCCGATGG

2051 TACCAGGGAG TTTCTGACTT TTGAAGTTCC ACTGAATGAC TCAGGATCTG

2101 CAGGGCTTGG TGTCAGTGTC AAGGGGAACC GTTCCAAAGA GAACCACGCA

2151 GATTTGGGGA TCTTCGTTAA ATCCATTATC AATGGTGGAG CTGCATCTAA

2201 AGATGGAAGG CTGAGGGTAA ATGACCAGCT GATAGCTGTG AATGGAGAAT

2251 CTCTACTGGG CAAAGCCAAC CAGGAAGCCA TGGAGACTCT ACGGAGGTCC

2301 ATGTCCACCG AGGGCAACAA GCGTGGCATG ATCCAGCTCA TTGTGGCGAG

2351 GCGGATCAGC AGATGTAACG AGCTGCGGTC TCCTGGGAGC CCTGCTGCAC

2401 CTGAGCTGCC CATCGAGACA GAACTGGATG ACCGAGAACG CAGGATCTCA

2451 CACTCCCTCT ACAGTGGGAT CGAGGGGCTG GATGAGTCGC CCACCAGGAA

2501 CGCAGCACTG AGCAGGATAA TGGCTGAGTC AGGAACATAG GATTTGCCAT

2551 ACTG

SEQ. ID. NO. 34

>gi|1666538|gb|AAB18670.1| par-3 gene product
MSASSTSSSSTSCPEGGEPSGSCKSSDEGESTLKKRMQQYGIASGYANSSISTLDRSQYQSLPLN
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LDMDDVLEEVFDLNYDQILAITDEANGGSTTPTYSQIQKQQHHYAQPLPYARKFDGGPSTPIAS
AFGSVTVNHQAHRAASPYNVGFARSNSRDFAPQPTHSKERRDSVVEVSSFDQIPQSGLRVSTP
KPSRQSEDVIDGKPMNQPILRSSLRTEASGSRTEEATPVKQSRVTLSPEVEKKLAEQDERKSER
RKHYDKNPGRFARGSDRKSRITDALLDARDRIADQLESQNPAEETKSQMIRVKIDQGPMPGTS
LVTRPPIPEKSENEKQLGIEVNAVFDESSELPGTSEPTKLSSVQIMKIEDGGRIAKDGRIRVGDCI
VAIDGKPVDQMSIIRVRASISDLAAVTSRPVTLIINRSLESFLEQESSAKPIQSALQQANTQYIGH
TTVVELIKSSNGFGFTVTGRETAKGERLFYIGTVKPYGVALGHLKSGDRLLEINGTPTGQWTQS
EIVEKLKETMVGEKIKFLVSRVSQSAIMSTSASSENKENEETLKVVEEEKIPQKLPLPALMTPPV
PKDTPALSPSGASRFEIVIPFINGSSSAGLGVSLKARVSKKSNGSKVDCGIFIKNVMHGGAAFKE
GGLRVDDRIVGVEDIDLEPLDNREAQAALAKKLKEVGMISSNVRLTISRYNECNPGQISRDLSR
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DGTSRNDESELPDSADPFNREGLGRKSLSEKRGMGAAADPQHIKLFQDIKHQRQNSAPTSSTQ
KRSKSQPRSSSQRNYRSPMKLVDLPTTAAASASTNSQNLDDSDMLNRRSQSMESINRPVESILR
GTGQIPTGSSSKVQFMQAASPDQHPFPPGAALLRLKNEESRSRDKSRRKSMGNPFSAMRNFFG
FGSKSRDASPEKTPTESVQLRSVERPKSIIDERNNGSSERAPPPLPPHQSQRRGSGGNVFVDYGE
PYGLIPQYPHNTTSGYESYADSELYDRYAAHRYHPRGGPIIDEDEYIYRQQSTSGNSPINTSSYV
NYGLPASNAYHVGSRIPPQTSSGSISKTSGAMRRVYPAEYDEDVAYHQQIPQQSTRYQQGSGS
GRGNADYHHMFNSWFAYTGGGAVGAAPVIKSSYGSSPVRIAAASAIERGESFVVEPVSGSSAS
ATDRRGRSTSSGAVASGSSSTGFQYAAKEKYADARSGKFNGGSTRLFIPRHGGGLSAAAFATN
FGGRAYETRGGGAGGSPSQYRRRDQGPPHRFPQY